

# **A Systematic Review of Survival Outcomes for HPV/p16 Sub-Groups of Oropharyngeal Cancer, And Drug Re-purposing In Head and Neck Cancer Cell Lines**

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## Abstract

Personalised cancer management has the potential to improve prognostic modelling and clinical outcomes, and to deliver tailor-made therapies. The aim of this study was, firstly, to conduct a systematic review of survival outcomes using a combination of HPV and p16 status in oropharyngeal cancers (OPC), with a particular focus on the significance of the discordant groups. Secondly, to investigate the drug re-purposing prospects of three drug targets (INH001, INH002, INH003) using HPV negative (SCC040) and HPV positive (VU147) head and neck cancer cell lines.

Two independent reviewers performed a systematic review of the literature. In total 1318 OPC cases were identified from the 7 included studies. Pooled analysis found the HPV+/p16+ group with the best prognostic outcomes, followed by the HPV-/p16+ cohort. The worst prognosis outcomes were found with HPV-/p16- followed by HPV+/p16-. The 5-year overall survival rates were 81%, 58%, 32% and 42% respectively.

The three drug targets were tested using proliferation, migration, flow cytometry and clonogenic assays. Both INH001 and INH003 significantly reduced proliferation of SCC040 cells at 72 hours compared to untreated control (74%  $\pm$  8.9 and 93%  $\pm$  0.4 respectively). Only INH003 significantly reduced proliferation of VU147 cells at 72 hours (86%  $\pm$  1.7). Similarly both drugs demonstrated a significant reduction in SCC040 cell survival using the clonogenic assay (99.5%  $\pm$  0.5 and 83%  $\pm$  8.7 respectively). INH003 also exhibited a significant effect on SCC040 cells in the flow cytometry cell cycle analysis.

These results demonstrate the hypothetical value of prognostic studies to increase our understanding of cancer pathophysiology and patient outcomes. Furthermore there is promise in drug re-purposing methodology to increase the availability of novel and effective cancer therapies.

**Dedicated to My Parents**

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*“No life ever grows great until it is focused, dedicated and disciplined”  
(Harry Emerson Fosdick)*

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## **Chapter 1: Introduction and Literature Review**

## 1.1 Head and neck cancers

### 1.1.1 Basic anatomy

The term head and neck cancer (HNC) refers to a heterogeneous group of malignancies, arising from any sub-site within the region but generally excludes brain tumours (MedlinePlus, 2014). Anatomical sub-sites include the oral cavity, nasopharynx, oropharynx, hypopharynx, thyroid, and larynx (Figure 1.1). Omitting thyroid and skin tumours, over 90% of HNC are squamous cell carcinoma (SCC) arising from mucous membranes (Suh et al., 2014). Thus they are usually referred to as HNSCC (head and neck squamous cell carcinoma).

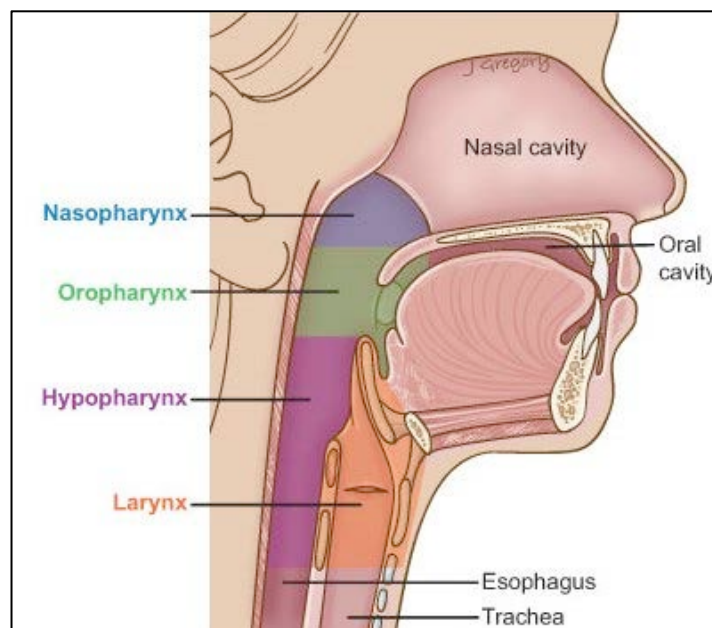


Figure 1.1: Anatomical sub-sites of head and cancers (THANC, 2015)

### **1.1.2 Epidemiology**

Worldwide incidence of HNC is estimated to be over 500,000 new cases per year (Jemal et al., 2011), with 300,000 related deaths (Boyle et al., 2008). Globally, it is the 5<sup>th</sup> most common cancer (Siegel et al., 2012), and ranked 8<sup>th</sup> in cancer-related deaths (Ragin et al., 2007a). There are considerable variations in incidence depending on both anatomical sub-site and geography.

In England the overall incidence of HNC has increased since 1990, according to a recent report (OCIU, 2010). However, the rates and trends between HNC types are variable, with considerable geographical differences. Between 1990 and 2006 the incidence of oral, oropharyngeal, palate, salivary gland and thyroid cancer has risen. The most significant increase has been observed with oropharyngeal cancer, which has more than doubled (OCIU, 2010). In contrast the incidence of nasopharyngeal and hypopharyngeal cancer has remained constant throughout this period. Laryngeal cancer is the only HNC type to have shown a reduced incidence of almost 20%. Despite there being common risk factors for all HNC types such as smoking and alcohol, there are some risk factors unique to certain subtypes. This may, in part, account for the variability in incidence rates.

Mortality rates from HNC have improved within the same period that has seen the increase in overall incidence (OCIU, 2010). This improvement in mortality is seen across the subtypes, however, oropharyngeal cancer mortality rates appear to have increased at a considerably slower rate than incidence. This suggests that while



incidence has increased significantly the modest increase in mortality rates may be attributed to improved disease management or changes in demographics profiles.

### **1.1.3 Risk factors**

There are various risk factors associated with HNC, with certain sub-types related to specific causes. Smoking and alcohol are associated with the majority of cases, and both have a synergistic effect (Maier et al., 1992). The change in incidence and mortality rates of HNC are likely to be related to these and other risk factor variations.

#### *1.1.3.1 Smoking*

In the 1950s several studies were published describing a link between smoking tobacco and lung cancers (Doll et al., 1950). Since then the role of smoking in cancer pathogenesis has been well established. Smoking is the major risk factor for all HNC subtypes (IARC, 2014). One study reported a risk of developing HNC that was greater than doubled for ever smokers compared to never smokers in patients that were also never drinkers (Hashibe et al., 2007). This association is linked in a dose-response manner according to quantity, duration and pack years. A large prospective study of 476,211 participants demonstrated a difference in HNC risk dependent on gender and smoking habits (Freedman et al., 2007a). Incidence rates of HNC for male never, previous and current smokers were 24.4, 36.9 and 147.3 per 100,000 person-years of follow up respectively. Rates for females were 4.8, 17.2 and 75.7 respectively. However, this study concluded that smoking is likely to influence HNC

pathogenesis more significantly in females than males; with smoking attributed to approximately 75% HNC cases in the female cohort, compared to 45% in men.

The risk of HNC appears similar among different forms of tobacco smoking, such as cigars and pipes. A recent study found patients who had smoked cigars but never cigarettes had a HNC odds ratio of 2.54 (Wyss et al., 2013), whereas pipe-smokers had an odds ratio of 2.08. The anatomical site of HNC can also vary according to the method of smoking. For example, reverse smoking (i.e. inserting lit end of cigarette into oral cavity) is associated with cancer of the hard palate (Van Der Eb et al., 1993).

The role of other smoking products, such as marijuana (cannabis), as risk factors in HNC is less clear. The use of marijuana has been associated with an increased risk of other cancers, such as lung malignancy (Callaghan et al., 2013). However, a case-controlled study conducted for HNC patients appeared to show no significant increase in cases, but this could not exclude a moderate effect (Berthiller et al., 2009).

#### *1.1.3.2 Chewing products*

The use of smokeless tobacco, such as chewing, is highly prevalent among South Asians (Abbas et al., 2014), and is a particular risk factor for oral cancer. A case-controlled study among a population in New England analysed the association between HNC rates and smokeless tobacco use (Zhou et al., 2013). Comparing ever users of smokeless tobacco to never cigarette users there was a significant increased

risk of cancer (odds ratio = 4.06). Similar to smoking tobacco, the risk was correlated to duration of use.

Non-tobacco chewing products related to HNC include betel nut. The betel nut seed, also known as areca nut, is derived from the areca fruit (DrugInfo, 2014). The seed is used to create betel quids, which is commonly chewed among the Asian population. A meta-analysis showed more than double increase in HNC cases in non-smokers when comparing betel nut users against non-users (Thomas et al., 2007). The betel nut is also sometimes combined with tobacco.

#### *1.1.3.3 Alcohol*

Alcohol is another major risk factor for HNC, and is dose-dependent. A pooled analysis of over 10,000 HNC cases demonstrated a doubling of risk among heavy drinkers (>3 drinks/day) who were never smokers (Hashibe et al., 2007). In a prospective questionnaire study, which included over 500,000 patients, analysis of cancer risk and alcohol consumption was performed (Freedman et al., 2007b). An interesting observation was that never drinkers had an increased incidence rate (per 100,000 person-years) of HNC (61.3 for males, 20.4 for females) compared to low-level drinkers (<1 drink/day; 34.4 for males, 14.7 for females). Thus this suggests a protective effect of alcohol at low consumption rates. However, among heavy drinkers (>3 drinks/day) HNC incidence was 77.6 for males, and 75.3 for females.

The synergistic effects of alcohol and tobacco are thought to be multiplicative rather than additive. In a multicentre and worldwide case-controlled study a moderate

increase in oral cancer risk was demonstrated in relation to consumption of alcohol or smoking alone (Castellsague et al., 2004). However, both alcohol and smoking in combination increased the rate of oral cancer 13-fold.

#### 1.1.3.4 Viruses

In recent years substantial evidence has emerged regarding the role of viruses in HNC pathogenesis. The most prominent example is human papillomavirus (HPV). Historically HPV has been strongly associated with cervical cancers and are classified as a sexual transmitted disease (CDC, 2014). In 1976 Professor Harald zur Hausen first suggested a link between papillomavirus and cervical cancer, later earning him a Nobel Prize in 2008 (zur Hausen, 1976). Infection with HPV is thought to account for >99% of cervical cancers, particularly high risk HPV (Walboomers et al., 1999). Other cancers related to HPV include oropharyngeal (Figure 1.2). In 1983 Syrjanen et al first described the relationship between oral cancer and HPV infection (Syrjanen et al., 1983).

Site	Percentage attributable to HPV infection	Percentage of which HPV16 and/ or 18
Cervix	>99	>75
Penis	47	74
Vulva, vagina	42	90
Anus	84	93
Mouth	16	95
Oropharynx	28	89

Figure 1.2: HPV-related cancers (PHE, 2014)

The relationship between HPV infection and risk of HNC is believed to be independent of alcohol and smoking history (Hennessey et al., 2009). In particular, it is oropharyngeal cancers (OPC) that have shown the most significant association with HPV infection. The role of HPV in these cancers will be discussed further (Section 1.2). HPV can be sub-classified into low and high-risk types. In both cervical and HNC the most prevalent HPV type is high-risk HPV16. This common subtype is likely transmitted between anatomical sites by sexual practices, such as oral sex. Regarding the change in incidence rates of HNC, particularly OPC, this may represent a change in sexual habits in the past few decades (Mercer et al., 2013). There is also substantial evidence of the prognostic value of HPV infection in HNC (Ragin et al., 2007a, Dayyani et al., 2010, O'Rorke et al., 2012).

The Epstein-Barr virus (EBV) has been implicated in the pathogenesis of nasopharyngeal cancers. EBV belongs to the herpesvirus family, and worldwide the majority of the adults are thought to be carriers (Thompson et al., 2004). In 1964 Sir Michael Epstein was the first to describe the association between EBV infection and cancer (Epstein et al., 1964). Infection with EBV is commonly associated with Burkitt's lymphoma, although not classified as a HNC tumour it can affect head and neck sub-sites. EBV is estimated to contribute to approximately 90% of nasopharyngeal cancers (Thompson et al., 2004). A retrospective review of HNC samples analysed the rate of EBV infection among sub-types of HNC, including oropharyngeal tumours (Goldenberg et al., 2004). Only 1% of samples were EBV positive, and 23% showed trace EBV DNA. Therefore it appears EBV does not play a prominent role in other HNC pathogenesis.

*1.1.3.5 Environmental factors*

There are several occupational and environmental risk factors related to HNC. The effect of exposure to wood dust among workers of wood-related industries was assessed during a meta-analysis of over 28,000 participants. There was an apparent increase in sinonasal and nasopharyngeal cancer rates among those exposed to wood dust, with a standardised mortality ratio of 3.1 and 2.4 respectively (Demers et al., 1995). In a retrospective study of Swedish construction workers, asbestos exposure doubled the risk of laryngeal cancer (Purdue et al., 2006). Furthermore the relative risk for pharyngeal cancer associated to cement dust was 1.9. Formaldehyde, a chemical used for industrial and household products, has been implicated in increasing risk of HNC. An Italian study analysed the association between 16 chemicals related to occupational hazards and risk of oral and oropharyngeal cancer. Formaldehyde appeared to increase the risk of oral cancers, with an odds ratio 1.8 (Merletti et al., 1991).

*1.1.3.6 Non-preventable factors*

Many of the risk factors previously mentioned are considered preventable. However, the risk of HNC can correlate in certain demographic subgroups. The risk of HNC is 3-fold higher in the male population (Langevin et al., 2011). This increase is partly attributed to the higher frequency of risk factors among males, such as smoking and drinking. Hormonal factors have been proposed as a potential cause for gender difference. Post-menopausal women using hormone replacement therapy were

found to have a decreased risk of HNC in one study, with more than a 50% reduction compared to controls (Langevin et al., 2011).

The incidence of HNC increases with age, with the majority of cases over 50 years old (Ridge, 2014). However, incidence rates appear to be increasing in the younger population (Golas, 2007), and this is thought to be due to the increasing role of HPV infection.

There is also a discrepancy in HNC rates between races, with a higher rate of laryngeal cancer among African-Americans (Ridge, 2014). Furthermore, survival appears worse among African-Americans (Molina et al., 2008), with a greater proportion of HPV negative HNC (Weinberger et al., 2010). Variations in risk factor exposure may account for these differences.

Genetic factors also contribute to increased risk of HNC. A meta-analysis demonstrated an increased risk of cancer among patients with a first-degree relative of HNC, with an odds ratio 1.7 (Negri et al., 2009). The risk appeared greater when the relative was a siblings rather than a parent. Several genetically related syndromes have increased incidence of HNC, such as Fanconi anaemia (Baez, 2008).

#### *1.1.3.7 Other risk factors*

Other potential factors can contribute to increasing HNC risk, such as variations in diet. A large meta-analysis performed by the INHANCE (International Head And Neck Cancer Epidemiology) consortium demonstrated an increased risk of

HNC relating to consumption of red and processed meats (Chuang et al., 2012). An improved survival was seen among cases with high intake of fruit and vegetables. There is also an association between nasopharyngeal cancer and consumption of salted fish, with a Chinese study showing the risk more than doubled (Jia et al., 2010).

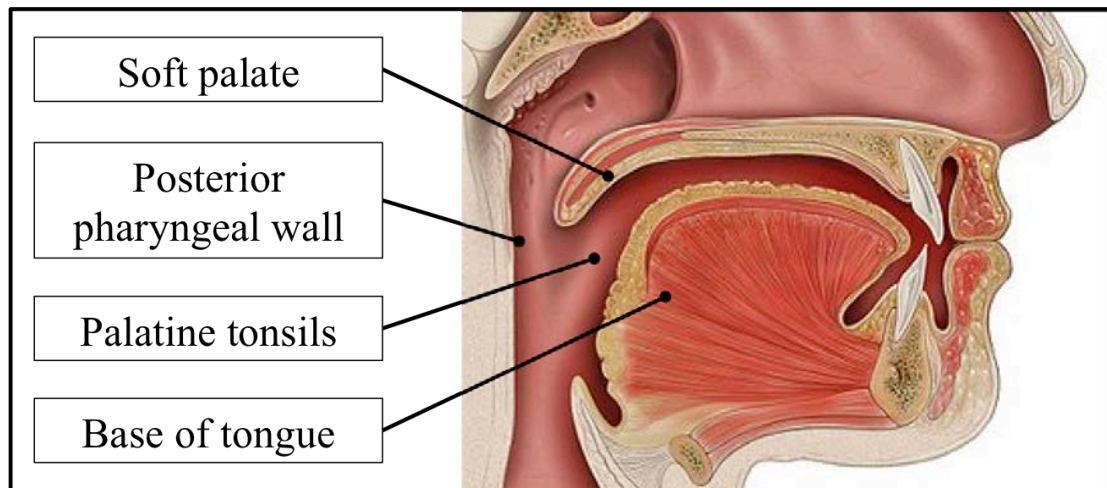
Immunosuppression can increase susceptibility to various cancers, including HNC. Organ transplant recipients receiving immunosuppressants have shown an increased risk of HNC. For example, in a recent meta-analysis of liver transplant recipients there was a 3.84 increase incidence of HNC (Liu et al., 2014).

## **1.2 Oropharyngeal cancer**

### **1.2.1 Anatomy**

The oropharynx is a three-dimensional structure defined by the anterior tonsillar pillar, circumvallate papillae, posterior pharyngeal wall, hard palate and the hyoid bone. The region is sub-divided into tonsils, base of tongue, soft palate, posterior and lateral pharyngeal walls (Figure 1.3).





*Figure 1.3: Illustration of oropharynx sub-sites (Lynch, 2006)*

### **1.2.2 Demographics and Trends**

Oropharyngeal cancer (OPC) has, and continues to show, a significantly increasing incidence. A comprehensive retrospective study was published in 2013 reporting on the incidence rate of OPC among 23 countries, from 1983 to 2002 (Chaturvedi et al., 2013). In total almost 70,000 cases of OPC were included in the analysis, the majority being male (54,700). The increasing incidence observed was mainly seen in developed countries such as USA, France, UK and Japan. In contrast there appeared to be non-significant or declining incidence in oral cancers among men in these countries, but an increase among women. Changing geographical and gender smoking practices is likely to account for the varying incidence rates, particularly in westernised societies, where the rate of smoking has decreased in recent years (Masironi et al., 1988).

As previously described, this global trend is also reflected in England. Here the incidence of OPC has more than doubled, from 1 in 100,000 to 2.25 in 100,000 population in the past few decades (Figure 1.4) (OCIU, 2010). This increase represents the most significant rise in any HNC type. A similar rise in OPC incidence has been evident in Scotland. Between 1989 and 1996 OPC incidence rate among males increased from 18 to 23.6 per 100,000, and 7.3 to 8.5 per 100,000 in females (Robinson et al., 2003). In other developed countries this pattern is similarly observed. In a Swedish study the incidence rate of tonsillar cancer from 1960 was examined, and observed a 2.6% increase per year in males and 1.1% increase in females (Hammarstedt et al., 2007).

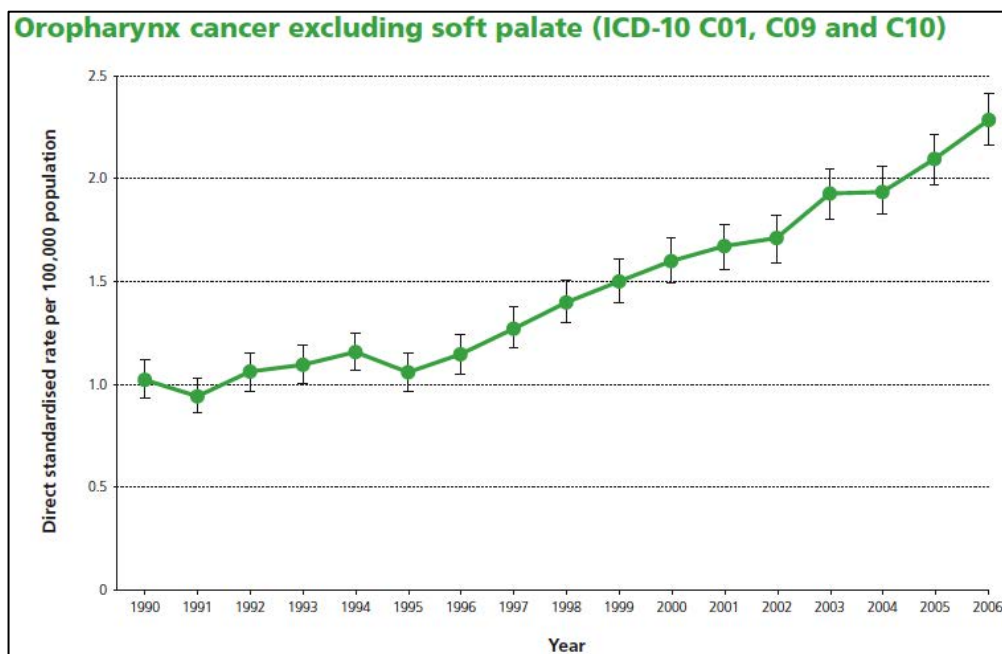


Figure 1.4: Illustration of increasing OPC incidence in England (OCIU, 2010)

Several studies have demonstrated that there is a particular increase in OPC cases among the younger population. Trends in OPC rates between 1992 and 2007 were examined in a Canadian study (Johnson-Obaseki et al., 2012). The median age of diagnosis of OPC decreased by 0.23 years/year over the study period, while other HNC types had an increasing median age. In addition, there was an improved survival rate for OPC of 1.5% per year in males. In a US study researchers found an increased incidence of tongue and tonsillar cancer between 1973 and 2001 among adults aged 20-44 years old (Shiboski et al., 2005).

Logically the length of exposure to traditional risk factors such as alcohol and smoking are reduced in the younger population, and therefore less likely to influence disease pathophysiology and incidence patterns. Instead trends in incidence appear to be related to the increasing role of HPV in the pathophysiology of OPC. The majority of HPV positive HNC tend to occur in the oropharynx. In a systematic review of 60 studies by Kreimer et al. there was an overall HPV prevalence of 25.9% in HNSCC (Kreimer et al., 2005). The greatest proportion of HPV positive cases was observed among the oropharyngeal cohort (35.6%), with HPV16 the most common subtype (86.7%). In a prospective study of 253 HNSCC there was a strong association of HPV positive tumours and oropharyngeal site, with an odds ratio 6.2 (Gillison et al., 2000). Prevalence rates appear to vary between studies, and are dependent on sub-sites of the oropharynx, particularly the tonsils and base of tongue. Analysis of 474 patients in a Swedish study demonstrated 79% prevalence of HPV in tonsillar cancers, and 75% in base of tongue tumours (Attner, 2013). Further evidence for HPV positive HNSCC appearing to be driven via a different pathophysiology comes from their association

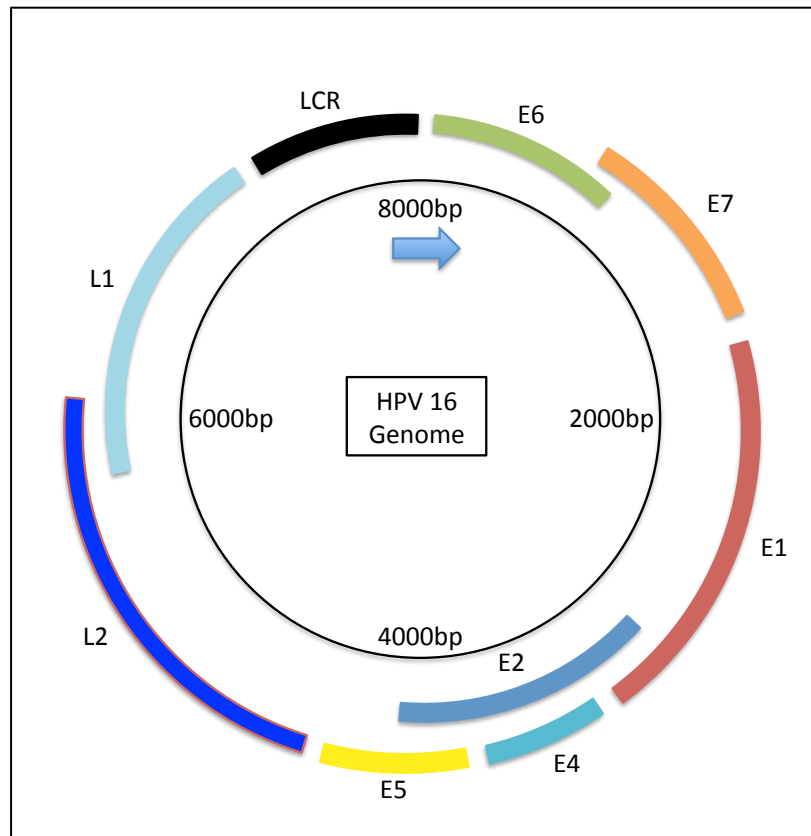
with patients that have low alcohol exposure, are non-smokers and have wild-type p53 gene expression (Gillison et al., 2000).

The prevalence of HPV in oropharyngeal cancers is thought to be attributable to changes in sexual practices and risk factor profile, and this might explain the typical demographics described. In one study the risk factors for high risk HPV among oropharyngeal cases included age<55 years, higher number of sexual partners, oral-genital sex and oral-anal sex (Smith et al., 2004). Furthermore there is evidence that early sexual contact increases risk of OPC (Heck et al., 2010).

### **1.2.3 HPV pathophysiology**

#### *1.2.3.1 Structure*

In 1907 an Italian doctor was the first to demonstrate the viral origin of common warts (Javier et al., 2008), subsequently found to be caused by human papillomavirus. The papillomavirus belongs to the Papoviridae family of viruses, which also include the polyomaviruses. The HPV virion is composed of an icosahedral structure measuring 55nm, and having no envelope (Fields et al., 1996). The virus is formed as a 8kb double-stranded DNA circular structure, contained within a protein capsid. Various viral genes are encoded within the genome, and subdivided as early and late (Figure 1.5). The virus infects only epidermal cells, in either skin or mucosal layers.



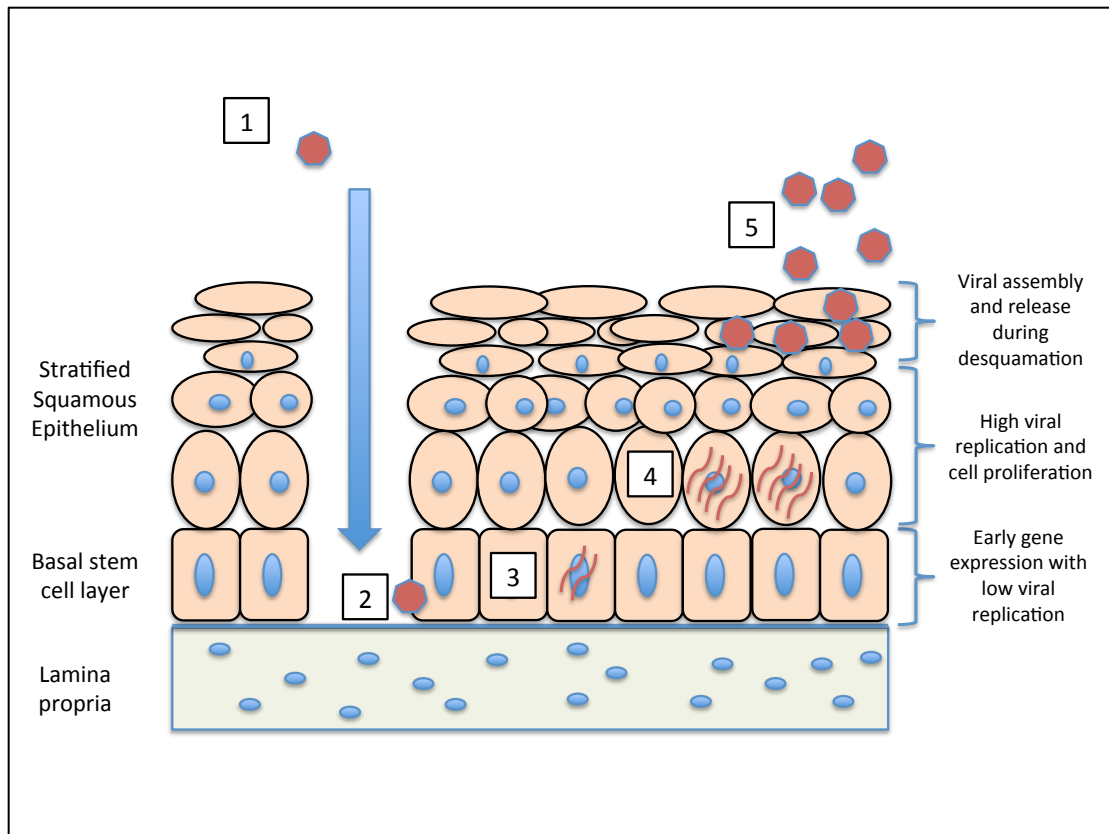
*Figure 1.5: HPV16 genome structure*

#### *1.2.3.2 Classification*

There are over 120 HPV types, which are classified into cutaneous and mucosal (Fernandes et al., 2013). Approximately 40 types are associated with mucosal infection. The HPV types are further classified as low or high risk, depending on their benign or malignant potential. Low-risk HPV include HPV-6 and HPV-11, and are associated with benign genital warts and laryngeal papillomatosis. The high-risk HPV, such as HPV-16 and 18, are associated with various cancers, in particular cervical cancer. The most common type among oropharyngeal cancer is HPV-16 (Kreimer et al., 2005).

*1.2.3.3 Pathogenesis*

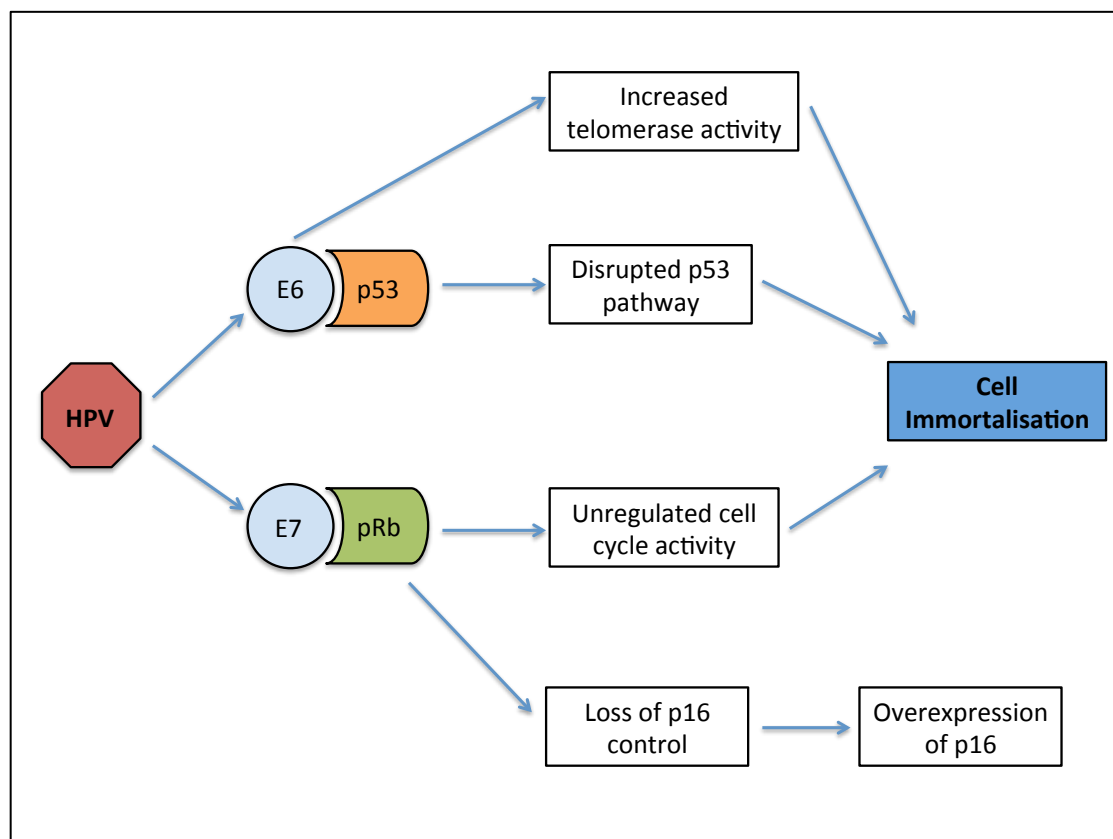
In order to infect epithelial cells the HPV virus must first enter the basal cell layer, which is likely achieved via small breaks in the skin or mucosa (Figure 1.6). The basal layer contains replicating stem cells that are essential for viral maintenance. The virus then enters an incubation period that can last weeks, months or years (Fernandes et al., 2013). Replication of the virus is considered in two stages (Munoz et al., 2006). First, the viral particles are maintained at a low count within the basal cell layer, thus evading the host immunity. The early viral proteins E1 and E2 contribute to the initial maintenance phase. During the life cycle of the epithelial layer the basal cells migrate towards the suprabasal layer and undergo terminal differentiation, with the virus entering the second stage of replication. Viral copy numbers increase dramatically in stage two, multiplying to thousands of viral copies per cell. The E4 and E5 viral proteins support replication and promote maturation of the virus.



*Figure 1.6: Life cycle of HPV- 1) HPV gains access to basal stem cells through areas of micro-trauma in the epithelial cell layer, 2) Entry into basal cells via endocytosis, 3) Maintenance phase with low viral replication, 4) Differentiation-dependent phase with high viral replication, 5) Complete HPV particles are released during normal epithelial desquamation*

The key oncoproteins in HPV pathogenesis are E6 and E7, working together to immortalise keratinocytes (Figure 1.7). E6 binds and inactivates p53, thus inhibiting apoptosis. Wild-type p53 acts as a tumour suppressor gene and is activated by DNA damage. The p53 functions include modulation of cell cycle and senescence (Scheffner et al., 1990). The E6 oncoprotein also induces telomerase activity by activating the hTERT promoter (Liu et al., 2009). E7 binds and promotes degradation of pRb (retinoblastoma protein), which is also a tumour suppressor gene. Normal pRb functions to block cell cycle progression from G1 to S-phase by binding to E2F factor (Giacinti et al., 2006). However, disruption of pRb by E7 promotes unregulated entry of cells into S-phase, with E7 also interacting directly with E2F transcription factor

(Fernandes et al., 2013). Cells entering unregulated S-phase tend to promote apoptosis via the p53 pathway. However, the E6 inactivation of p53 prevents apoptosis and allows abnormal cells to persist. The process of cell immortalisation accumulates abnormal mutations and chromosomal instability, and eventually can lead to cancer formation. The late genes L1 and L2 code for the viral capsid. Once the virus has matured and assembled they are released during the normal desquamation of epithelial cells. The life cycle of the virus ensures there is little interaction with the host immunity. Eventually most infected individuals clear the papillomavirus, but persistence can vary according to the HPV type (Bulkmans et al., 2007).



*Figure 1.7: Illustration of HPV E6 and E7 interactions with host cell pathways*



#### 1.2.3.4 *Diagnosis*

Detection of HPV in oropharyngeal cancer can be performed by either direct or indirect methods. Direct methods include polymerase chain reaction (PCR), in-situ hybridisation (ISH), antibody detection and Southern blot assay (Venuti et al., 2012). The most common techniques are PCR and ISH. The most common indirect method is detection of p16 by immunohistochemistry (IHC).

The PCR method of detection also includes reverse transcriptase PCR (RT-PCR). These methods are highly sensitive for HPV detection, but have several limitations. PCR techniques can be time and resource consuming. They also have reduced specificity, and are unable to distinguish between episomal and integrated HPV. However, emerging new PCR techniques have shown improved specificity to be able to detect HPV active disease.

In contrast to PCR the ISH method of HPV detection is highly specific, but is limited by low sensitivity. While PCR requires a very low number of viral DNA for detection, positivity with ISH is estimated to require at least 10 viral copies per cell (Venuti et al., 2012).

Numerous studies have included p16 IHC as an indirect detection method of HPV-related tumours. This method utilises monoclonal antibodies against p16<sup>ink4a</sup>, with positivity calculated according to staining patterns. However, there are several definitions for positive staining, which include various percentages (e.g.  $\geq 70\%$ ) of p16 staining within the tumour sample. This technique is also dependent on the

observer. Previous studies have suggested algorithms for accurate detection of HPV infection in HNSCC samples, combining p16 IHC with direct HPV detection methods (Smeets et al., 2007).

Several studies have demonstrated a strong correlation between HPV status and p16 expression. In a study by Ang et al. 93% of HPV positive OPC cases were p16 positive; defining p16 positivity as  $\geq 70\%$  strong and diffuse nuclear and cytoplasmic staining (Ang et al., 2010). In contrast the HPV negative OPC cohort were positive for p16 in 18.8%. In a prospective study comparing HPV ISH and p16 IHC detection methods the authors discovered 100% of HPV-16 HNSCC tumours were positive for p16, using the  $\geq 70\%$  cut-off point (Singhi et al., 2010). There were also 24% HPV negative cases showing p16 positivity. A recent meta-analysis in 2014 investigated the correlation of HPV and p16 overexpression among oropharyngeal cancer cases, with a total of 39 studies included (Gronhoj Larsen et al., 2014). The studies differed according to HPV (PCR and/or ISH) and p16 detection methods, including various p16 staining cut-off points. The reviewers compared the sensitivity of p16 correlation to HPV status according to three p16 cut-off points;  $\geq 70\%$ , 5-70% and verbal definition only. The highest correlation of p16 to HPV appeared within the studies using  $\geq 70\%$  cut-off, with a combined sensitivity of 0.927. Studies with 5-70% cut-off had a sensitivity of 0.894, and verbal only was 0.791.

#### *1.2.3.5 The role of p16*

The p16 gene (INK4A) was first discovered as a cyclin-dependent kinase (CDK) inhibitor in 1993 (Serrano et al., 1993). The gene is located on chromosome

p21 and codes for a 16kDa protein product. The CDK family are important factors in normal cell cycle control. Therefore, considerable research has focused on CDK inhibitors, such as p16.

p16 protein functions to prevent G1 to S-phase transition within the cell cycle via interaction with the pRb pathway (Romagosa et al., 2011). Initially p16 binds to CDK4 or 6, thereby preventing the formation of cyclin D-CDK4/6 complex (Figure 1.8). This complex normally functions to phosphorylate pRb factors. The resultant hypo-phosphorylated Rb protein continues to bind and inactivate E2F transcription factor. E2F transcription factor regulates genes involved in DNA replication, and therefore inhibition by hypo-phosphorylated pRb leads to cell cycle arrest and proliferation.

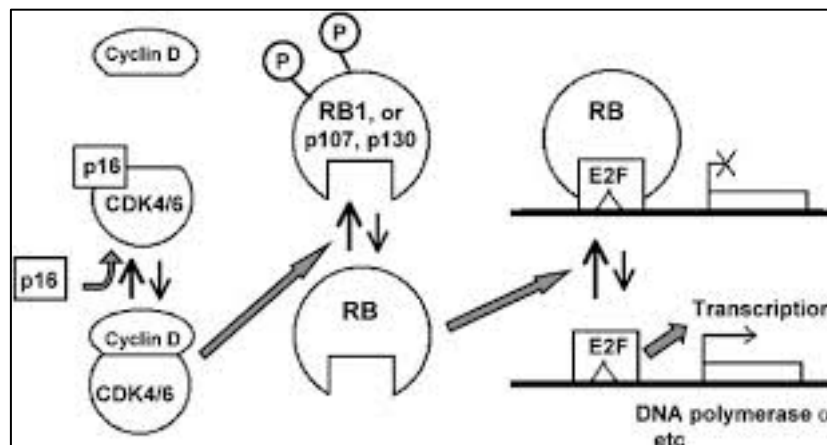


Figure 1.8: p16-pRb pathway and cellular interactions (Bennett, 2003)

The pathophysiological mechanism of HPV-induced cancers appears to occur via disruption to the p16-pRb pathway. While E7 expression inactivates pRb function, there is an effect on the regulation of p16 by disruption of the normal negative regulation by functional pRb (Hara et al., 1996). Therefore, one expects an overexpression of p16 in HPV positive cancers (Figure 1.7). Furthermore,

overexpression of p16 can vary by cellular location between nuclear and cytoplasmic. The role of p16 within the cytoplasm is not clearly understood (Romagosa et al., 2011).

The importance of p16 in cancer pathogenesis is demonstrated by the frequency of deletions, mutations and methylation of p16 among several malignancies. Within head and neck cancers there is a reported average of 50% homozygous deletions, 10% mutations and 20% methylation of the p16 gene (Liggett et al., 1998).

#### **1.2.4 Prognostic biomarkers**

The World Health Organisation (WHO) defines a biomarker as “*any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease*” (WHO, 2011). The use of cancer biomarkers is well illustrated with one of the most well established being HER2 detection in a proportion of breast cancer cases. Those tumours expressing HER2 tend to be more aggressive than HER2 negative disease (Madell, 2014). The availability of this biomarker also allows for targeted therapy against those tumours expressing HER2, and treatments such as Herceptin have been shown to improve survival in HER2 positive breast cancer. Similar biomarkers have been investigated for oropharyngeal cancers, particularly HPV and p16 positivity.

#### 1.2.4.1 HPV

There is clear evidence from multiple studies demonstrating improved prognosis of HPV positive compared to HPV negative HNSCC. An early study by Gillison et al. demonstrated a 40% increased overall survival and 59% improved disease-specific survival in HPV positive HNSCC (Gillison et al., 2000). In an analysis of 60 primary tonsillar cancers there was an improved 3 and 5-year survival rate between HPV positive cases compared to HPV negative (Mellin et al., 2000). The 3-year survival was estimated at 65.3% and 31.5% respectively, with 5-year survival 53.5% and 31.5% respectively. A further study on tonsil cancers also reported on improved recurrence and survival outcomes in HPV positive tumours (Li et al., 2003).

Additional studies have reported on survival outcomes in OPC with respect to HPV positivity. Researchers investigating the prognostic role of HPV in oral and oropharyngeal cancers demonstrated a significantly better overall survival when compared to HPV negative cancers (73% vs. 35%), as well as disease-specific survival (79% vs. 45%), with HPV the most reliable prognostic marker in OPC (Klozar et al., 2008). A retrospective study of OPC cases found a statistically significant improvement in overall and disease-free survivals, as well as locoregional control, in the HPV positive cohort (Sedaghat et al., 2009). In a US study of cancer registries there was a significant difference in median survival between HPV positive and negative OPC cases of approximately 131 and 20 months respectively (Chaturvedi et al., 2011).

A seminal paper on HPV and survival in oropharyngeal cancer was derived from the RTOG-0129 study data by Ang et al, involving a multi-centre research collaboration (Ang et al., 2010). The RTOG-0129 study was a randomised-controlled phase III trial comparing two regimens of concurrent radiotherapy and chemotherapy in advanced HNSCC. Between 2002 and 2005 a total of 743 patients were enrolled into the trial, with 60.1% (433) oropharyngeal cancer cases. The HPV status was determined for 323 OPC cases, with 63.8% testing positive for HPV by ISH. The overwhelming majority of HPV positive OPC tested positive for HPV-16 (96.1%). Investigation of survival outcomes using Kaplan-Meier analysis revealed 3-year overall survival in HPV positive OPC was 82.4%, with HPV negative at 57.1%. Similarly the rates for progression-free survival were 73.7% and 43.3% respectively. Multivariate analysis, adjusting for various confounders, estimated a 58% reduction in risk of death in the HPV positive cohort. The researchers then performed recursive-partitioning analysis and found HPV status to be the most significant determinant of survival, followed by number of pack-years smoking, nodal stage (HPV positive only) and tumour stage (HPV negative only). They used these 4 factors to divide oropharyngeal cancer cases into three separate risk groups; low, intermediate and high. The 3-year overall survival rates in these groups were 93%, 70.8% and 46.2% respectively (Figure 1.9). This example of risk stratification offers a potential use in both the research and clinical setting.

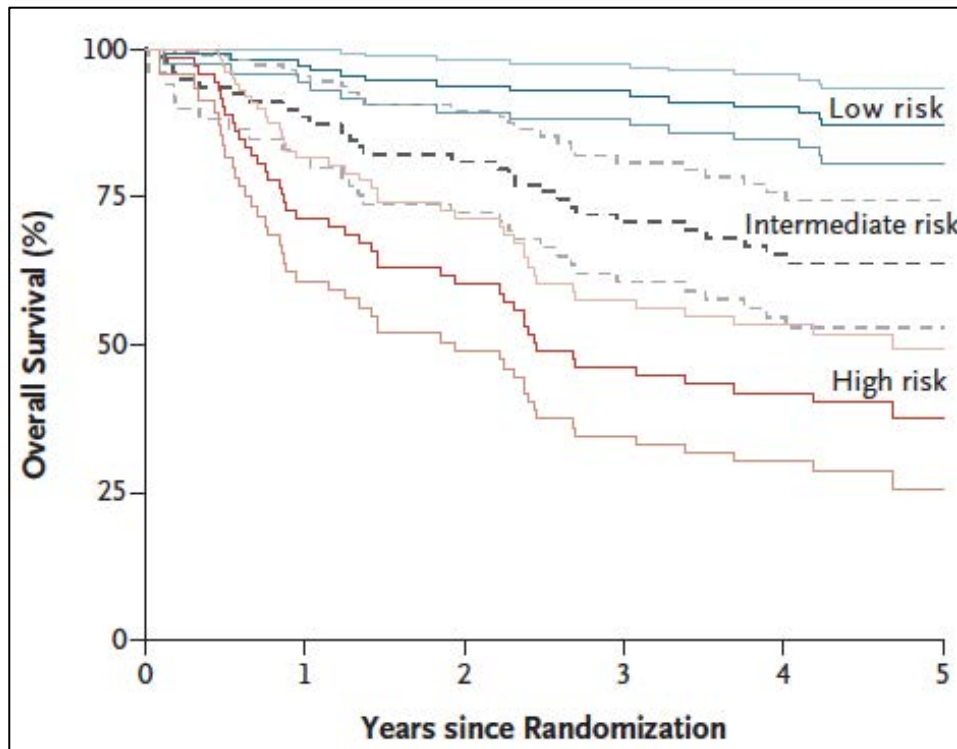


Figure 1.9: Kaplan-Meier curves and 95% confidence intervals according to risk stratification of oropharyngeal cancer cases (Ang et al., 2010)

In recent years there have been several systematic reviews and meta-analyses assessing the prognostic value of HPV in HNSCC. An early review by Ragin et al. included 37 studies, with patient number per study ranging from 22 to 254 (Ragin et al., 2007b). Twelve studies contained site-specific data. Analysis of all HPV positive HNSCC cases demonstrated an overall improvement in survival of 15% compared to HPV negative cases. This effect was even greater in a subset analysis of oropharyngeal cancer cases, with an improvement in disease-free survival of 49%, and overall reduction in risk of death of 28% in HPV positive patients.. A further review published in 2010 included a total of 5681 HNSCC cases from 34 studies, with an overall HPV prevalence of 22% (Dayyani et al., 2010). Meta-analysis showed an improved survival of 59% in HPV positive HNSCC, and sub-group analysis of oropharyngeal cases was 60%. A more recent review by O'Rorke et al. examined a total of 42 studies (O'Rorke et al., 2012). Improved overall survival (54%) in HPV

positive HNSCC was demonstrated by meta-analysis. Pooled hazard ratios for oropharyngeal cancers indicated a 53% reduction in risk of death. This effect size was later confirmed in another meta-analysis of prognostic markers in OPC, which included 18 studies related to HPV. An improvement in overall survival of 57% was seen in HPV positive cases (Rainsbury et al., 2013).

#### 1.2.4.2 *p16*

As described, the pathophysiological mechanism of HPV infection indirectly interacts with p16 expression via inactivation of pRb (Section 1.2.3.2). Correlation of HPV status and p16 appears highly sensitive. Therefore p16 testing has been suggested a surrogate marker of HPV-related oropharyngeal cancers (El-Naggar et al., 2012, Langendijk et al., 2010).

The detection of p16 in oropharyngeal cancers has also shown prognostic ability. One study investigated the prognostic significance of p16 in 123 oropharyngeal SCC, and found improved survival parameters (Weinberger et al., 2004). Compared to patients with normal p16 expression, those with over expression (>80% tumour staining) had an 83% reduced risk of local recurrence, along with improved disease-free survival (64%) and improved overall survival (58%). Over expression of p16 remained an independent prognostic indicator in multivariate analysis. In a more recent study of 102 OPC the 5-year survival rate for p16 positive ( $\geq 5\%$  tumour staining) cases was 59.3%, compared to 24.5% in the p16 negative cohort (Fischer et al., 2010). The tumour status of p16 appeared to be the strongest prognostic marker in multivariate analysis. Within the RTOG-0129 study (Ang et al.,



2010) p16 status was used to stratify cases. The authors reported 3-year overall survival of 83.6% in the p16 positive group and 51.3% in p16 negative group, and 3-year progression-free survival rates of 74.4% and 38.4% respectively. Adjusted p16 hazard ratio of death was 0.33. The meta-analysis by Rainsbury et al. included 6 studies on p16 and survival, with a pooled relative risk of 0.43 in p16 positive cases ( $p < 0.00001$ ). Among the included studies the cut-off for p16 positivity ranged from 25-70% (NB- 2 studies did not specify cut-off value), but the results remained significant after sensitivity analysis.

#### *1.2.4.3 Other oropharyngeal biomarkers*

The majority of studies reporting on oropharyngeal cancer and survival have utilised detection of HPV DNA and/or p16 staining as diagnostic and prognostic biomarkers. There has been increasing interest in antibody detection methods related to HPV infection. In a study by Kreimer et al. a cohort of HNSCC were tested for pre-diagnosis biomarkers of HPV, including 135 OPC cases. HPV-16 E6 antibody was detected in 34.8% of OPC pre-diagnosis plasma samples, but only 0.6% in control samples (Kreimer et al., 2013). The increase in E6 plasma level was detected more than 10 years prior to diagnosis. In a recently published meta-analysis prognostic biomarkers were investigated, including HPV, p16, p53 and HIF1 $\alpha$  (Rainsbury et al., 2013). While high-p53 level was correlated with improved overall survival, this was not statistically significant. Only HPV and p16 demonstrated statistically significant improvement in overall survival. Various other biomarkers were also identified from selected studies, but a meta-analysis could not be performed due to the small study sizes.

A multicentre trial (PredicTr-OPC), co-ordinated by InHANSE (Institute of Head and Neck Studies and Education), is currently underway to investigate numerous potential prognostic biomarkers in oropharyngeal cancers.

### 1.2.5 Discordant groups

As demonstrated, a strong improvement in survival is seen in HPV positive HNSCC compared to HPV negative, particularly oropharyngeal SCC. Furthermore, the majority of HPV positive tumours also show p16 positivity, while HPV negative tumours are more likely to show p16 negativity. Both HPV and p16 are independent markers of improved prognosis. However, there remain two so-called discordant groups where HPV and p16 do not correlate, HPV+/p16- and HPV-/p16+. By utilising data from existing studies it is possible to calculate proportion of oropharyngeal cancers belonging to four subgroups; HPV+/p16+, HPV-/p16-, HPV+/p16- and HPV-/p16+. Several studies examples are included in Table 1.1.

<b>Study reference</b>	<b>HPV+/p16+</b>	<b>HPV-/p16-</b>	<b>HPV+/p16-</b>	<b>HPV-/p16+</b>
(Ang et al., 2010)	59%	29%	2%	7%
(Klusmann et al., 2003)	47%	47%	6%	0%
(Hafkamp et al., 2008)	40%	53%	1%	6%
(Abbas et al., 2014)	50%	43%	4%	3%

*Table 1.1: Example studies with proportion of oropharyngeal cases per subgroup*

These studies demonstrate that while the two discordant groups represent a minority of cases, they are not insignificant. There are conflicting reports regarding

the prognostic significance of these two groups. A total of 239 oropharyngeal cases were analysed in a retrospective study by Lewis et al. (Lewis et al., 2010). The authors concluded that there appeared to be no difference in survival rates between HPV+/p16+ and HPV-/p16+, and suggested p16 testing alone for risk stratifying cases in future. However, Perrone et al. reported results contradicting this (Perrone et al., 2011). This study indicated similar reduced overall survival between HPV-/p16- and HPV-/p16+. To date no adequate study or systematic review has addressed the prognostic significance of these subgroups. This subject will be addressed in this thesis.

### **1.3 Current treatments in Head and Neck cancers**

The management of head and neck cancers is dependent on several factors including tumour site, histological type, pathological grade and TNM stage. Treatment is also dependent on whether the tumour is primary, secondary or recurrent disease. Patient-related factors comprise of age at presentation, premorbid status, co-morbidities and patient's preferences. The approach to management of these cancers will involve a multi-disciplinary team involving surgeons, specialist nurses, pathologists, oncologists, radiologists and various allied health professionals.

The aim of treatment is disease control and improved prognosis, while preserving the functional ability of involved organs. Numerous modalities and regimes exist at present for the management of HNC. The common treatment types will be discussed in further detail.

### 1.3.1 Surgery

#### 1.3.1.1 *Primary tumour*

The intention of surgery is to preserve organ function while ensuring complete removal of the tumour with a clear histopathological margin. Depending on factors such as site and size of the tumour, several techniques may be employed. These include tonsillectomy, pharyngectomy, laryngectomy and tracheostomy. Surgical techniques can involve open, endoscopic, robotic and laser surgery. Reconstructive surgery maybe required if extensive excision is undertaken in order to preserve or regain function.

Locoregional tumour control can be can also be achieved by radical radiotherapy. The Scottish Intercollegiate Guidelines Network (SIGN) recommends non-surgical treatment if survival rates are similar between both modalities (SIGN, 2006). In a randomised controlled trial comparing surgery and adjuvant radiotherapy with concurrent chemoradiotherapy in 119 HNSCC cases (stage III/VI non-metastatic disease) no significant difference in disease-free survival rates between treatment arms was detected (Soo et al., 2005). Surgical outcomes can depend on the ability to achieve clear margin. In a retrospective cohort study 261 HNSCC patients were treated with primary surgery, with 89% demonstrating negative surgical margins (Haque et al., 2006). There was almost a 3-fold increase in all-cause mortality in cases with positive margins compared to negative. Cases with positive margins were more likely to present as advance disease (stage IV), and with a larger tumour size (>2cm).

In cases of locoregional recurrence surgery most often offers the best chance of salvage, if technically possible. Decisions regarding salvage operations depend on previous treatments and likely benefits to patient, along with patient choice, with improving survival the primary goal. A meta-analysis of 32 studies estimated an overall 5-year survival of 39% in HNSCC cases treated with salvage surgery (Goodwin, 2000).

Palliative surgery can be employed in advanced HNSCC or cases unsuitable for primary treatment modalities. The aim is to improve symptom control, and can involve debulking procedures and tumour embolisation.

#### *1.3.1.2 Neck surgery*

A large proportion of HNSCC will have locoregional metastases to cervical lymph nodes at presentations. Surgical removal of these involved nodes via neck dissection procedures can be utilised. Historically, radical neck dissection (RND) was first established and involves removal of ipsilateral cervical lymph nodes from level I to V (Subramanian et al., 2006). Non-lymphatic structures are also excised in RND, which include sternocleidomastoid muscle, internal jugular vein and spinal accessory nerve. Extended neck dissection involves RND with additional removal of lymph nodes and/or non-lymphatic structures. However, in modified (functional) radical neck dissection there is preservation of one or more of the non-lymphatic structures. More recently, a change in the nomenclature and approach to removal of nodal disease has led to increasing use of more selective neck dissection procedures. The

principle is to excise certain lymph node groups according to predicted patterns of metastasis, dependent on primary tumour site.

HNSCC can present with no apparent neck involvement, clinically or radiologically. However, risk of occult lymph node disease can vary between sub-sites. For example, oropharyngeal SCC has a reported >50% risk of pathological occult nodal metastasis in node negative disease (clinically and radiologically), compared to <15% in glottic disease (SIGN, 2006). The SIGN guideline recommends prophylactic neck treatment in node negative disease if the risk of occult nodal metastasis is >20%, with either neck dissection or radiotherapy.

#### *1.3.1.3 Side effects and complications*

Surgical treatment is likely to affect functional ability of various head and neck organs, and is of course dependent on the type of surgery performed. Generally speech, breathing and swallowing can be temporarily or permanently affected. Post-operative management can be challenging and may require a multidisciplinary input. The burden of disease involves physical and mental factors, which must be addressed to ensure optimal disease outcomes.

Complications associated with surgery can be defined as general or specific, immediate or early or late. Every operation is related to specific complications and can include nerve injury, vascular injury or flap-related difficulties. For example, pharyngocutaneous fistula (PCF) can occur following a total laryngectomy. In one study 246 consecutive patients undergoing total laryngectomy were investigated, with

16% developing post-operative PCF (Redaelli de Zinis et al., 1999). Risk factors for PCF formation include postoperative haemoglobin <12.5g/dl and preoperative radiotherapy (Paydarfar et al., 2006).

### **1.3.2 Radiotherapy**

#### *1.3.2.1 External beam radiotherapy*

Radiotherapy (RT) utilises high-energy ionising radiation to treat primary or nodal disease. The use of RT can be used as a single treatment modality for curative intent (radical RT) in early disease. Alternatively RT can be combined with surgery (adjuvant RT) or chemotherapy (chemoradiotherapy), or as palliative treatment (palliative RT). Delivery of radiotherapy can be achieved using various regimes involving fractionated treatment. Conventional fractionation involves delivery of 1.8-2Gy daily doses for 5 days per week, over a 6-week period.

Modified (altered) fractionation comprises of variations in daily radiation dose or number of fractions as either hypo, hyper or accelerated fractionation. Hypofractionation is an increased daily radiation dose delivered over a shorter period of time. Hyperfractionation therapy increases the number of dose fractions, while accelerated radiotherapy delivers a greater rate of radiotherapy over a shorter period. The purpose of the different strategies is to achieve a compromise between disease control and treatment toxicity. Numerous studies relating to these radiotherapy regimes have been published, and appear inconclusive. A study comparing conventional, hyperfractionated and accelerated therapy in HNSCC concluded there

was no significant difference in disease response or survival between these treatments (Krstevska et al., 2006). Likewise the RTOG study found similar survival rates between the accelerated and conventional treatment cohorts, both combined with chemotherapy (Ang et al., 2010). However, a meta-analysis of 15 trials appeared to show altered fractionation improved overall survival by 8% compared to conventional radiotherapy (Bourhis et al., 2006).

Recent advances in radiotherapy include proton therapy and 3D radiotherapy. There is increasing interest in a new form of radiotherapy known as Intensity Modulated Radiotherapy (IMRT). Using high-precision 3D conformal radiotherapy and computer inverse planning allows a greater dose of therapy delivery with reduced toxicity to surrounding tissues (Taylor et al., 2004). However, this method can be time and resource intensive with unknown long-term results. A recent review by O'Sullivan et al. found no significant difference in prognostic outcomes between 2D radiotherapy or 3D/IMRT treatment (O'Sullivan et al., 2012). Nonetheless IMRT appeared to reduce the rate of xerostomia and osteoradionecrosis in the included studies, as well as improve quality of life.

#### *1.3.2.2 Brachytherapy*

An alternative to external beam radiotherapy is brachytherapy, which involves localised and internal treatment of tumours. This method is reserved for accessible tumours, such as oral and oropharyngeal cancers. The theoretical advantage of brachytherapy is precise high-intensity radiation delivery with minimal surrounding tissue toxicity, with the main disadvantage being lack of access to tumours.



#### *1.3.2.3 Side effects*

The use of radiotherapy can avoid or minimise the role of surgery, and thus maintain functional ability of organs. However, radiation therapy is associated with significant early and late side effects that can be debilitating. The severity of these complications is dependent on intensity and duration of therapy. Early side effects include nausea, vomiting, dysphagia, radiation-induced dermatitis, temporary xerostomia and mucositis. In a systematic review of HNSCC treated with radiotherapy the average rate of mucositis was 80% (Trotti et al., 2003). Long-term complication can include on going dysphagia (sometimes requiring permanent gastrostomy feeding), secondary malignancies, osteoradionecrosis and permanent xerostomia.

### **1.3.3 Chemotherapy**

#### *1.3.3.1 Current evidence*

In the management of head and neck cancers chemotherapy is used in combination with radiotherapy, rather than a single modality. Neoadjuvant treatment refers to a chemotherapy course prior to definitive surgery or radiotherapy, while adjuvant therapy is delivered after such procedures. Concurrent chemotherapy relates to combined treatment with radiation. The benefit of chemotherapy in HNC is debatable. In a randomised phase III trial comparing locoregional treatment (surgery and/or radiotherapy) with or without neoadjuvant chemotherapy in non-metastatic

HNSCC, there appeared no significant difference in overall survival between the treatment arms (Zorat et al., 2004). However, neoadjuvant therapy only improved survival among the inoperable cases. A meta-analysis study was performed to assess the effect of additional chemotherapy with locoregional treatment in non-metastatic HNSCC (Pignon et al., 2000). Overall survival improved by 4% at 2 and 5 years with additional chemotherapy. Furthermore this benefit was a result of improved survival in concurrent treatment, with no advantage seen with neoadjuvant or adjuvant therapies. Current SIGN guidelines recommend concurrent chemoradiotherapy with Cisplatin, rather than radiotherapy alone, in cases with locally advanced non-metastatic HNSCC (oral, oropharynx, larynx, hypopharynx) when organ preservation is intended or the primary tumour is unresectable (SIGN, 2006). Depending on tumour site and stage concurrent chemoradiotherapy can also be utilised with or without surgical management. Neoadjuvant and adjuvant therapies are not recommended in combination with surgery alone.

Multiple chemotherapy agents exist, and can be used in various combinations. Cisplatin is the most commonly used chemotherapy drug in head and neck cancers. Other agents include Docetaxel, Carboplatin, 5-fluorouracil (5-FU) and Methotrexate. Previous and current trials have investigated Cisplatin therapy with additional drugs. For example, the TAX 323 and 324 trials investigated the potential benefit of neoadjuvant Docetaxel in combination with Cisplatin and 5-FU in the treatment of HNSCC. The TAX 323 phase III trial recruited 358 patients with advanced non-resectable HNSCC, comparing neoadjuvant Cisplatin and 5-FU therapy with or without additional Docetaxel, followed by radiotherapy or concurrent chemotherapy depending on disease progression (Vermorken et al., 2007). There was significantly

improved progression-free and overall survival in the treatment arm containing Docetaxel. The TAX 324 phase III trial recruited 501 patients with advanced HNSCC that were either non-resectable or candidates for organ preservation therapy. The same neoadjuvant treatment arms were used as for TAX 323, but were followed by concurrent chemotherapy. Similarly the overall survival rate was significantly improved in the Docetaxel treatment arm (Posner et al., 2007).

#### 1.3.3.2 Cisplatin

Cisplatin was first discovered in 1844 by an Italian chemist named Michele Peyrone, and thus termed Peyrone's chloride (Lebwohl et al., 1998). However, it took over a century for the anti-proliferative effects of Cisplatin to be observed by Barnett Rosenberg. Initial trials showed promising results in testicular and ovarian tumours. In the late 1970s the drug was approved in the USA and worldwide for the treatment of various tumours, including head and neck cancers.

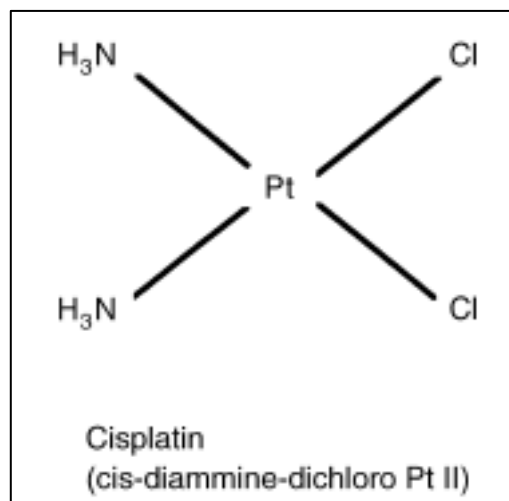


Figure 1.10: Illustration of Cisplatin molecule (Lebwohl et al., 1998)

Cisplatin is an inorganic platinum-based compound belonging to the transition metal chlorides (Figure 1.10). It acts as an alkylating agent to induce target cell death

by several mechanisms. The drug induces oxidative stress by increasing reactive oxidative species, which leads to DNA damage. Alkyl groups also attach to DNA bases causing fragmentation and also preventing DNA replication. Cross-linking of DNA between bases occurs and there is increased mismatching of nucleotides, leading to mutations. Various signalling pathways are activated, including p53, causing cell cycle arrest. Eventually these mechanisms initiate apoptosis (Dasari et al., 2014).

#### *1.3.3.3 Side effects*

The vast majority of current chemotherapy agents are non-specific, and thus have numerous side effects. Cancer cells are generally more susceptible to treatment due to instability and abnormal replicating patterns. However, all cells within the body can be affected. Depending on the replication cycle of cells, early and late side effects can occur. Early symptoms may include nausea, vomiting, mucositis, end-organ damage and hair loss. Damage to haemopoietic cells can lead to anaemia, thrombocytopenia and neutropenia. Therefore there is an increased risk of infection and neutropenic sepsis. Late side effects can include permanent end-organ damage, neuropathy, infertility and secondary malignancies.

#### **1.3.4 Targeted molecular therapy**

In recent years there has been increasing research and interest in molecular targets for treating head and neck cancers. Theoretically such therapies can improve disease outcomes with more favourable side effects profile. An example is Cetuximab

(Erbix), which was initially licenced for locally advanced HNSCC in combination with radiotherapy. Currently the license has been extended for recurrent or metastatic HNSCC in combination with platinum-based chemotherapy (EMA, 2014).

Epidermal growth factor receptors (EGFR) belong to the ErbB family of tyrosine kinase receptors, which are involved in cell signalling pathways (Bonner et al., 2006). There is evidence that EGFR is over expressed in HNC cells, and therefore an ideal target for molecular therapy (Grandis et al., 1993). Cetuximab is a monoclonal antibody that specifically binds to EGFR to disrupt the signalling pathways, and thus prevent cell division.

In a multinational randomised trial 424 patients with locoregional advanced HNSCC were recruited into two treatment arms; either radiotherapy alone or concurrent radiotherapy with Cetuximab (Bonner et al., 2006). There was significantly improved locoregional control, progression-free survival and overall survival in the Cetuximab group. This has led to recommendations from both SIGN and NICE for concurrent Cetuximab and radiotherapy use in locally advanced HNSCC who are unsuitable for concurrent chemoradiotherapy (SIGN, 2006, NICE, 2008). In another multicentre phase III randomised trial 442 patients with untreated recurrent or metastatic cancer were enrolled (Vermorken et al., 2008). One arm received platinum-based chemotherapy with fluorouracil, and the other the same treatment with the addition of Cetuximab therapy. The results showed significantly improved progression-free and overall survival in the treatment arm containing Cetuximab.

As with other therapeutic agents, there are associated side effects with Cetuximab. These include skin reactions (e.g. rash), mucositis and deranged liver function tests (EMA, 2014).

### **1.3.5 Novel therapies**

There is no doubt that advancements in understanding and management of head and neck cancer has led to improved survival outcomes. However, improving prognosis cannot be wholly attributed to more effective treatment strategies. The increasing role of HPV in disease pathogenesis has been proven to demonstrate favourable survival, irrespective of treatment modality. Furthermore, changes in survival rates have been modest or unchanged in the past 3 decades in developed countries. While early stage disease appear to show excellent long-term prognosis, advanced or metastatic HNC continue to demonstrate poor survival. Therefore both low and high risk HNC present unique challenges. Although long-term survival is of paramount importance to patients, this must be balanced with maintaining a reasonable quality of life. There is recent interest in de-escalating treatment, with the purpose of maintaining treatment outcomes while reducing long-term toxicity. Currently there are several de-escalation trials. An example is De-ESCALaTE HPV, which is an international randomised phase III trial, being led by Warwick medical school. The aim of this study is to compare the outcomes between Cisplatin and Cetuximab, both combined with standard radiotherapy in HPV positive oropharyngeal cancers.

Besides Cetuximab there has been no major drug discovery in head and neck cancer treatment since the 1970s, with the advent of chemotherapy. The panacea drug of HNC treatment would ideally demonstrate high efficacy with minimal side effects. In order to achieve this target researchers and pharmaceutical companies continue to explore various avenues for drug discovery. However, traditional drug discovery is a time-consuming and expensive process (Figure 1.11). Drug development can take anywhere between 10-20 years (average 12 years) from preclinical testing to approval (Dickson et al., 2004). The Association of British Pharmaceutical Industry puts an estimate average cost of drug development at £1.15 billion (Triggle, 2014). There is also a high failure rate, which can occur at any stage during the process, even after drug approval. An alternative to this method of drug discovery is drug repurposing, which will be discussed in further detail.

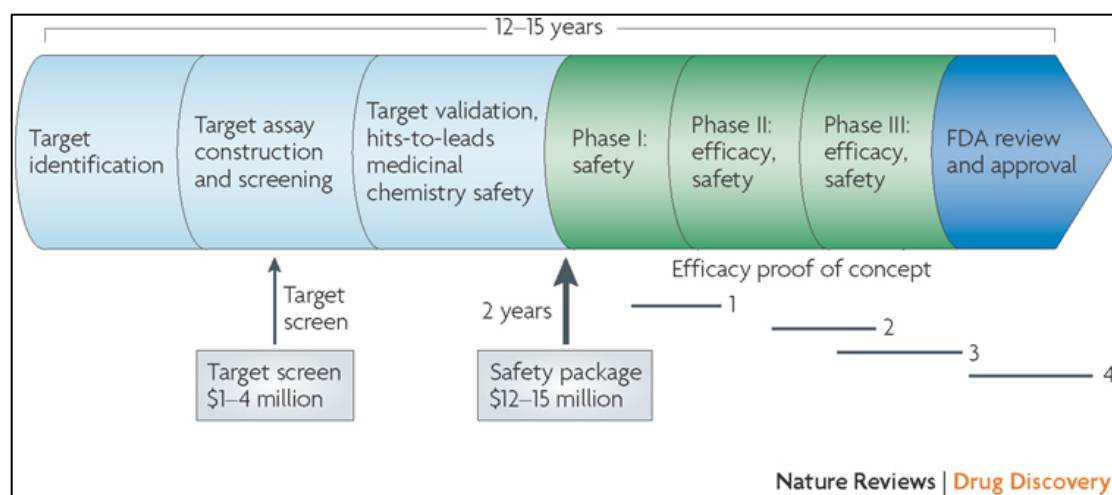


Figure 1.11: Illustration of drug development timeline (Roses, 2008)

## 1.4 Drug repurposing

### 1.4.1 Background

Drug repurposing, also known as drug re-positioning, is the process of finding new therapeutic uses for existing drugs. The theoretical advantages of this method of drug discovery are reduced timescale and expense. While any agent can be a potential target for drug repurposing, the costs can be further reduced by utilising off-patent medications. Currently there are several commercial drug libraries that can be utilised for the purpose of high throughput screening. One such method has been described as the SOSA approach (selective optimisation of side activities), by selectively testing existing drugs on new targets (Wermuth, 2006). Established agents will have a well-known side effects profile and proven safety in human models. Similarly, the pharmacokinetic and pharmacodynamic properties would also have been elucidated. Therefore drug targets identified from such screening could potentially be utilised in the clinical setting in a far shorter timescale.

A famous example of drug repurposing is Viagra (Sildenafil). Initially Viagra was developed for the treatment of angina. The drug inhibits PDE5 (phosphodiesterase) and therefore prevents cGMP breakdown (Ghofrani et al., 2006). cGMP is involved in various cell-signalling pathways including mediation of smooth muscle relaxation, thus leading to vasodilation. However, volunteers of early studies reported several side effects, including penile erection. Although angina treatment was the initial target of this agent, eventually the drug was repurposed. In 1998 both the FDA and European Agency approved Viagra for the treatment of erectile



dysfunction. In 2005 it was also approved for the treatment of pulmonary hypertension (Ghofrani et al., 2006).

#### **1.4.2 Cancer therapy**

There are numerous examples of drug repurposing for cancer treatments. In the 1950s Thalidomide was introduced to treat morning sickness in pregnant women (Kim et al., 2011). However, over the course of several years devastating teratogenic side effects were observed in the babies of some mothers, leading to a ban in 1961. Further studies of Thalidomide have described the mechanism of action, including inhibition of angiogenesis. Renewed interest in this drug has led to repurposing of Thalidomide in the treatment of leprosy and multiple myeloma (Singhal et al., 1999).

Commonly used drugs have also shown promise in cancer therapy, including Aspirin, Simvastatin and Metformin. In a systematic review of prophylactic Aspirin use there appeared to be a decrease in cancer-related incidence and mortality of several cancer types, particularly colorectal and oesophageal malignancy (Cuzick et al., 2015). Despite the relative increased risk of adverse events, such as gastrointestinal bleeding, the benefits appear to outweigh the harms. Metformin is commonly used in the treatment of non-insulin dependent diabetes. There is growing evidence demonstrating a reduced incidence rate of various cancers with Metformin use. One meta-analysis reported a reduced incidence rate of malignancies in diabetics treated with Metformin including colorectal, hepatocellular, prostate and breast cancers (Noto et al., 2012). Overall cancer incidence risk was reduced by 33%, and cancer-related mortality risk by 34%.

### **1.4.3 The AcceleraTED platform**

Development of novel therapies in head and neck cancers is the main aim of the AcceleraTED project (Accelerated Translation of therapeutic agents and Emerging Developments in Head and Neck and Thyroid cancer). Utilisation of different drug libraries and robotic high-throughput screening has identified several drug targets. As part of this thesis three of these drug targets have been investigated further (INH001, INH002, INH003).

## **1.5 Aims and Objectives**

While head and neck cancers are relatively uncommon compared to other malignancies there is an increasing incidence rate worldwide, with a significant shift in disease pathogenesis. The role of HPV in HNSCC is becoming more prominent, particularly in oropharyngeal cancers. Several prognostic indicators and biomarkers have been proposed for risk-stratification. Combining these biomarkers can potentially improve prognostic predictions and guide management. Furthermore, current treatment regimes appear to have almost reached maximum survival benefits. There is a constant need for novel therapies that can improve outcomes while minimising toxicity. Drug repurposing is a promising method of rapidly increasing available treatments, and has not been systematically investigated in head and neck cancers. Ultimately the aim is to improve outcomes by personalised treatments, combining prognostic studies and novel therapies.

The main objectives of this project are:

- To perform a systematic review of survival outcomes in subgroups of oropharyngeal cancers according to a combination of HPV and p16 status. In particular the prognosis among discordant groups will be analysed, and pooled analysis performed.
- To investigate 3 drug targets as potential drug repurposing agents in head and neck cancer treatments. Differences between HPV positive and negative cell lines will also be examined.

## **Chapter 2: Systematic Review and Descriptive Analysis**

## 2.1 Introduction

The human papillomavirus (HPV) belongs to a group of viruses with over 100 genotypes (Gearhart, 2014). They have been associated with epithelial tumours, particularly high-risk genotypes such as HPV 16 and 18. Cancers linked to HPV infection include cervical, vaginal, anal, and oropharyngeal (NCI, 2012).

Head and neck cancer (HNC), including thyroid cancers, are the 5<sup>th</sup> commonest cancer group worldwide (Siegel et al., 2012). Within this group squamous cell carcinoma (SCC) accounts for over 90% of cases, excluding thyroid and skin tumours (Schiff, 2013). A recent report of HNC incidence in England shows that oropharyngeal SCC (OPSCC) rates have more than doubled since 1990, which is more than any other HNC sub-site (OCIU, 2010). This is likely to be due to a change in sexual behaviour and risk factor characteristics, with an increasing role of HPV in disease pathogenesis.

There has been recent interest in the relationship between HPV and HNC, particularly with respect to survival and disease-free outcomes. Systematic reviews have demonstrated improved survival in HPV positive patients, particularly in OPSCC (Ragin et al., 2007b, Dayyani et al., 2010, O'Rorke et al., 2012). Further studies of HPV in OPSCC have focused on various prognostic biomarkers, such as p16 and p53 (Rainsbury et al., 2013). The p16 gene codes for a tumour suppressor protein (INK4A), and has been suggested as a possible surrogate marker of HPV infection (Chen et al., 2012, Dayyani et al., 2010). HPV-related oropharyngeal tumours appear to show p16 protein overexpression, via mechanisms previously

described (Chapter 1). Furthermore, p16 positive HNC show improved survival outcome (Rainsbury et al., 2013).

While HPV and p16 appear to show favourable prognosis independently, the prognostic combination of HPV and p16 to predict survival is unclear. A landmark study by Ang et al. risk stratified HPV OPSCC into 3 survival groups (low, intermediate, high risk), by a combination of HPV status, smoking and tumour staging (Ang et al., 2010). The study found that 93.2% of HPV positive cases were p16 positive, and 80.3% of HPV negative cohort was p16 negative. However, 3.4% were HPV+/p16- and 18.8% HPV-/p16+. These latter two sub-groups represent discordant results, with unknown clinical significance.

A literature search, including CRD (Centre for Reviews and Dissemination) databases, found no current systematic review reporting survival outcomes according to these 4 sub-groups, particularly the significance of the two discordant groups. The aim of this systematic review was to determine the prognostic outcomes of OPSCC by analysing combinations of HPV and p16. These include the following:

- HPV+/p16+ (i.e. true positives)
- HPV-/p16- (i.e. true negatives)
- HPV-/p16+
- HPV+/p16-

## 2.2 Materials and Methods

Training for the systematic review was undertaken using the InHANSE guidelines (Mehanna, 2011), and supplemented with other written articles (CRD, 2009, Chung et al., 2006, Khan et al., 2003, Ressing et al., 2009). Prior to commencing the systematic review a protocol was devised, and approved by the review supervisors (Professor Hisham Mehanna and Jayne Wilson).

### 2.2.1 Search strategy

#### 2.2.1.1 Search Terms

In order to identify appropriate studies search terms were selected as Boolean operators, as follows:

‘HPV’ or ‘human papillomavirus’ or ‘papillomavirus’ or ‘p16’

AND

‘head’ or ‘neck’ or ‘oral’ or ‘buccal’ or ‘mouth’ or ‘tongue’ or ‘oropharyngeal’ or ‘tonsil’ or ‘palate’ or ‘pharyngeal’ or ‘pharynx’ or ‘hypopharynx’ or ‘larynx’ or ‘laryngeal’

AND

‘cancer’ or ‘neoplasm’ or ‘tumour’ or ‘SCC’ or ‘squamous cell carcinoma’

AND

‘recurrence’ or ‘prognosis’ or ‘mortality’ or ‘survival’ or ‘outcome’

The main anatomical head and neck sub-sites were included in the search terms in order to ensure no potential study was excluded.

### *2.2.1.2 Identifying studies*

Identification of relevant studies was performed, and repeated by a second independent reviewer (Nicola Graham, InHANSE). Initial searches were completed on 31<sup>st</sup> January 2014, using OvidSP (Wolters Kluwer Health, NY, USA). This included the following databases:

- Ovid Medline (R) In Process and Other Non-Indexed Citations and Ovid Medline (R)- 1946 to Present (National Library of Medicine, USA)
- Embase Classic and Embase- 1947 to Present (Elsevier, AMS, NED)

A further search was completed using the Cochrane Library (The Cochrane Collaboration). References from searches were all exported into EndNote X7 software (Thomson Reuters, Version X7.0.1). Study duplicates were removed automatically by EndNote on exporting, and using the 'Find Duplicates' feature of the programme.

Unpublished studies (i.e. grey areas of literature) were searched using the Web of Science databases (Thomson Reuters, USA). These include conference proceedings (1990-Present), Science Citation Index (1900-Present), BIOSIS citation index (1969-2008), and SciELO citation index (1997-Present). The ProQuest database (Ann Arbor, MI, USA) was examined for any relevant dissertations or thesis. Finally, relevant



conference websites were searched for abstracts, including AHNS (American Head and Neck Society) and ASCO (American Society of Clinical Oncology).

Study selection was then commenced (Section 2.2.2), and reference lists of included studies searched for any further references. Review articles were also searched for relevant references. An updated search of OvidSP was performed on the 29<sup>th</sup> December 2014 to identify any recent studies since the initial search.

### **2.2.2 Study selection**

Selection of relevant studies was performed independently by both reviewers. A trial of study selection was completed with 10 studies. Any disagreements were resolved by discussion, and if not resolved then the study was included into the next stage of selection and discussed with the review supervisors.

The following inclusion criteria were chosen for initial study selection:

- Human studies only
- Head and neck SCC, any site
- Report prognostic outcomes according to HPV and/or p16
- All study types (observational and experimental, retrospective and prospective)
- Any language

Titles were searched using the inclusion criteria, and the databases from both reviewers were merged. The number of agreed studies was calculated by EndNote, by the removal of duplicates.

All included studies were further analysed by assessing the abstracts, and using the following inclusion criteria:

- Oropharyngeal sub-sites only (base of tongue, tonsil etc.)
- Report on prognostic markers according to HPV and p16 in combination

Excluded articles included non-oropharyngeal cancers, reviews, editorials or opinions, and insufficient data. Reasons for exclusions were documented. Full papers of selected articles were then collated. Papers with no institutional access were located by the University of Birmingham library, and direct email contact with authors.

### **2.2.3 Quality assessment**

In order to assess quality of included studies a quality assessment tool was adapted from a previous systematic review (Rainsbury et al., 2013). Each paper was scored according to 6 domains (Table 2.1), based on potential biases (Hayden et al., 2006). Studies were scored 0-2, depending on the number of criteria met within each domain (0 = none, 1 = partial, 2 = all), with a total of 12 points. Both reviewers performed quality assessment independently, and an average score calculated.

Domain
<b>1. <u>Study participation</u></b> <ul style="list-style-type: none"> <li>• Study population described</li> <li>• Recruitment described</li> <li>• Inclusion and exclusion criteria</li> <li>• Adequate participation</li> <li>• Baseline population described</li> </ul>
<b>2. <u>Study attrition</u></b> <ul style="list-style-type: none"> <li>• Adequate response rate</li> <li>• Information about drop outs</li> <li>• No important difference between included patients and drop outs</li> </ul>
<b>3. <u>Prognostic factor measure</u></b> <ul style="list-style-type: none"> <li>• Clear definition of prognostic factors measured</li> <li>• Cut-off points included</li> <li>• Method of measure accurate and reliable</li> <li>• Adequate proportion of study population have complete data</li> <li>• Method same for all samples</li> </ul>
<b>4. <u>Outcome measure</u></b> <ul style="list-style-type: none"> <li>• Clear definition of outcome measured, including period of follow up</li> <li>• Method of outcome accurate and reliable</li> <li>• Method same for all samples</li> </ul>
<b>5. <u>Confounding measure</u></b> <ul style="list-style-type: none"> <li>• All important confounders measured</li> <li>• Clear definitions of confounders</li> <li>• Measure of confounding accurate and reliable</li> <li>• Confounders accounted in study design and analysis</li> </ul>
<b>6. <u>Analysis</u></b> <ul style="list-style-type: none"> <li>• Sufficient data for analysis</li> <li>• Adequate statistical models used</li> <li>• No selective reporting of results</li> </ul>

Table 2.1: Quality assessment tool

#### **2.2.4 Data extraction**

Full papers were reviewed and relevant data extracted. This data was input into a pre-defined spreadsheet (Microsoft Excel for Mac 2011 Version 14.0.0),. These included the following fields:

- Study details (Author, Title, Language etc.)
- Quality assessment score
- DNA extraction method
- HPV detection (PCR/ISH)
- p16 detection and cut-off points
- Patient demographics for each sub-group (age, sex etc.)
- Oropharyngeal sub-sites
- TNM staging
- Treatments
- Prognostic outcomes (e.g. overall survival)

All data extraction was performed by one reviewer, and validated by the second reviewer. Any disagreements were resolved by discussion.

### 2.2.5 Statistics

Following data extraction the spreadsheet results were examined with both reviewers and supervisor. Prior to statistical analysis the data was discussed with a biostatistician (Ikhlaaq Ahmed, CRCTU, University of Birmingham) to determine if a meta-analysis was appropriate. In case of insufficient data for meta-analysis a descriptive analysis would be performed. To statistically measure inter-rater reliability, Cohen's Kappa score was also calculated.

## 2.3 Results

### 2.3.1 Study selection

#### 2.3.1.1 PRISMA flow diagram

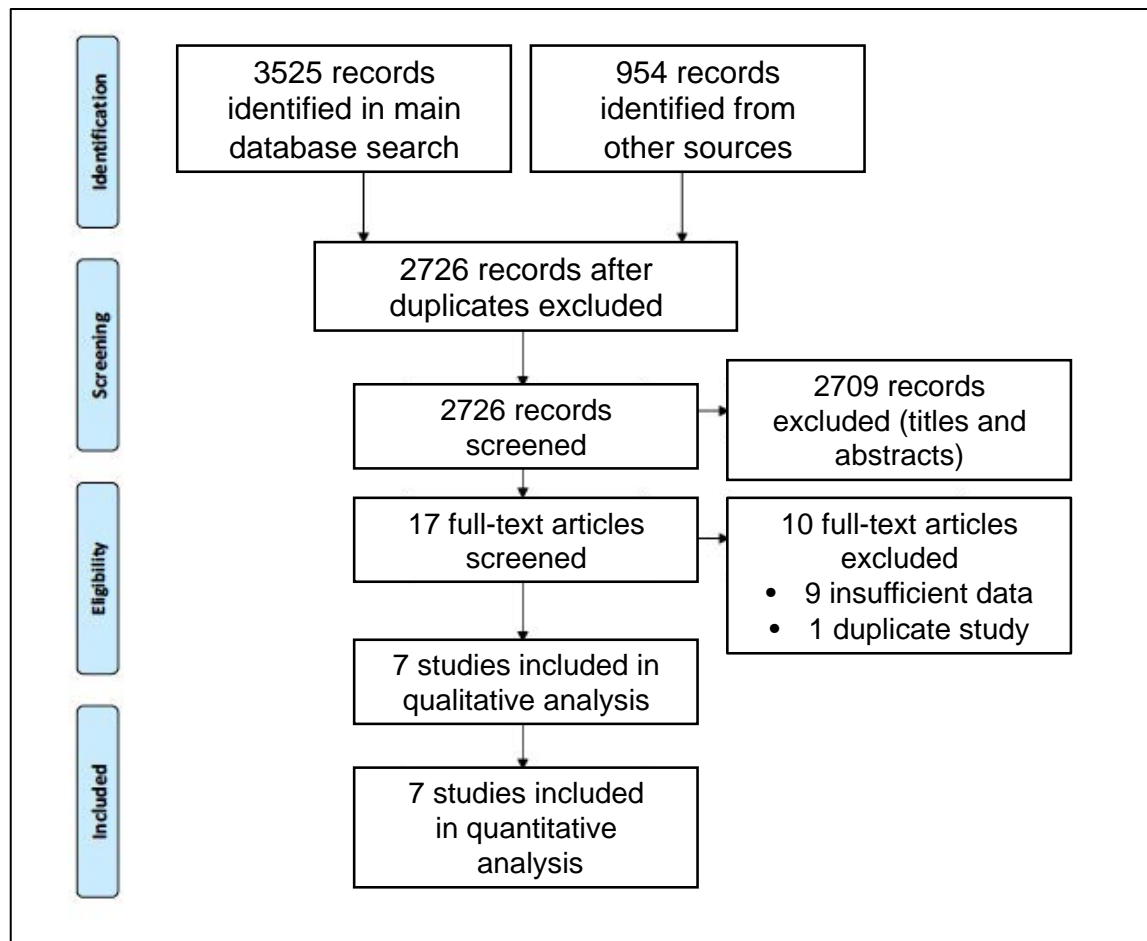


Figure 2.1: PRISMA flow diagram representing the stages of study selection

The results are presented according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist (Liberati et al., 2009). The main database searches included Medline, EMBASE and Cochrane library, and yielded 3525 studies (Figure 2.1). A further 954 records were identified from grey areas of literature searches, as described in section 2.2.1.2. Reference lists of included studies were examined, but no other relevant studies were found.

### *2.3.1.2 Excluded studies*

After the initial searches were completed all duplicates were removed using the EndNote software, leaving 2726 potential studies. Both reviewers screened study titles independently. The results of the title screening from both reviewers were combined and 682 records remained. Studies were excluded if the titles were obviously unrelated to the head and neck cancers, and did not focus on HPV and/or p16 prognostic indicators. Following screening of titles the abstracts were further analysed according to the initial exclusion criteria, and yielded 279 potential studies.

The abstracts underwent a second screening phase to select studies related to oropharyngeal cancers only, with 140 studies remaining. At this stage the final inclusion criteria was applied, and studies selected if they related to OPSCC and prognostic indicators described according to HPV/p16 combination. In total 17 studies were selected for full paper screening. Reasons for excluding abstracts are detailed in Table 2.2.

Reasons for Exclusion	Number of Studies
Insufficient data	54
No HPV data	47
No p16 data	11
Abstract only available	4
Review article	2
No access to paper*	2
Irrelevant	
• Cancer stem cell markers and survival	1
• Molecular characterisation of HPV-/p16+ OPC	1
Editorial	1

*Table 2.2: Reasons for excluded abstracts in second abstract screening phase.*

*\*Unavailable via The University of Birmingham and British Library services*

The 17 full papers were screened and a further 10 studies were excluded, with the majority due to insufficient data and one appearing to be a study with duplicate data. In total 7 studies were included in the qualitative and quantitative analysis.

### *2.3.1.3 Inter-rater reliability*

In order to assess degree of agreement between both reviewers Cohen's Kappa testing was applied (Cohen, 1960). This statistical test takes into account agreements that could occur by chance alone, and is calculated as follows:

$$\kappa = [\text{Pr}(a) - \text{Pr}(e)] / [1 - \text{Pr}(e)]$$

The  $\text{Pr}(a)$  value represents the actual agreement observed, and  $\text{Pr}(e)$  the expected agreement value. The results of the title screening performed by each reviewer is summarised in Table 2.3.



		Reviewer 2 (NG)		
		Yes	No	Total
Reviewer 1 (MJ)	Yes	283	216	499
	No	183	2044	2227
	Total	466	2260	2726

Table 2.3: Summary of title screening by both reviewers

Calculating the Kappa value as follows:

$$\text{Pr}(a) = (283+2044) / 2726 = 0.854$$

$$\text{Pr}(e) = [(466 \times 499 / 2726) + (2227 \times 2260 / 2726) / 2726] = 0.709$$

$$\therefore \kappa = (0.854 - 0.709) / 0.291 = 0.498$$

A value of 1 represents perfect agreement between two raters, and 0 an agreement no better than that expected by chance alone. However, the Kappa value is only one such method to represent inter-rater agreement, with various arbitrary systems to define its significance, as seen in Table 2.4. According to this scale the inter-rater agreement appears to be moderate at the title screening stage.

Kappa value range	Significance of agreement
<0	Poor
0-0.2	Slight
0.21-0.4	Fair
0.41-0.6	Moderate
0.61-0.8	Substantial
0.81-1	Almost perfect

Table 2.4: Significance of Kappa score, adapted from Landis et al (Landis et al., 1977)

In Table 2.5 the results of abstract screening by both reviewers is summarised, and the Kappa value is as follows:

		Reviewer 2 (NG)		
		Yes	No	Total
Reviewer 1 (MJ)	Yes	214	22	236
	No	43	403	446
	Total	257	425	682

*Table 2.5: Summary of first abstract screening by both reviewers*

$$\text{Pr(a)} = (214+403) / 682 = 0.905$$

$$\text{Pr(e)} = [(257 \times 236 / 682) + (425 \times 446 / 682) / 682] = 0.538$$

$$\therefore \kappa = (0.905 - 0.538) / 0.462 = 0.794$$

It appears the inter-rater agreement score improved to substantial during the abstract screening stage.

### 2.3.2 Included studies

#### 2.3.2.1 Descriptive summary

The systematic search yielded 7 full papers included in the qualitative and quantitative analysis. These are summarised in Table 2.6.

Reference	Study type	Country	Language	Total number of patients in study	Number included in review analysis
(Evans et al., 2013)	Retrospective cohort	Wales	English	147	138
(Hong et al., 2013)	Retrospective cohort	Australia	English	647	647
(Junor et al., 2012)	Retrospective cohort (2 cohorts)	Scotland	English	Cohort 1= 118 Cohort 2= 136	Cohort 1 = 77 Cohort 2 = 103
(Lewis et al., 2010)	Retrospective cohort	USA	English	239	209
(Song et al., 2012)	Retrospective cohort	South Korea	English	56	47
(Stephen et al., 2012)	Retrospective cohort	USA	English	80	20
(Weinberger et al., 2006)	Retrospective cohort	USA	English	107	77

*Table 2.6: Summary of included papers*

The included studies were published in the period from 2006 to 2013. All these papers were designed as retrospective cohort studies and written in English. In total 42% (3 studies) were conducted in the USA, and 29% (2 studies) in the UK.

The first study by Evans et al, conducted in South Wales, identified cases of oropharyngeal cancer diagnosed between 2001 and 2006 using pathology databases. The aims of the authors were to investigate prevalence of HPV in OPC and their link to clinical significance. They collected demographic and outcome data by searching electronic health records, patient notes, and direct contact with General Practitioners. In each case a fresh frozen paraffin-embedded (FFPE) sample block was retrieved and two pathologists confirmed the histological diagnosis of OPC. In total 147 cases of OPC were obtained for analysis, which represented 83% of cases diagnosed within the study period. However, only 138 FFPE blocks contained sufficient tumour for further HPV analysis.

Hong et al identified 647 cases of OPC treated with curative intent, and investigated the significance of the discordant groups. Cases were collected from 10 Australian centres diagnosed in the period of 1979 to 2009. Their inclusion criteria were availability of tumour samples and patient-related data and outcomes, which were obtained from institutional databases. The number of excluded cases was not mentioned.

The study by Junor et al was conducted in Scotland using population-based databases from a regional referral centre. The focus of the paper was the change in treatment of OPC between two cohorts. Cohort 1 was derived from a database of OPC cases diagnosed from 1999 to 2001, with the preferred treatment surgery +/- postoperative radiotherapy or radiotherapy alone, and neck dissection performed prior to radiotherapy for N2/3 disease. In Cohort 2, with OPC cases diagnosed from 2003 to 2005, the treatment of choice was concurrent chemotherapy and radiotherapy, with neck dissection performed post-therapy only if a complete response had been achieved.

Lewis et al investigated the significance of p16 status in OPC cases, particularly the discordant p16+/HPV- group. In total they identified 239 OPC cases from the Washington University databases diagnosed from 1997 to 2008.

The study by Song et al was the only Asian-based research identified for inclusion in this review, and was conducted in South Korea. The authors identified 56 cases of tonsillar SCC diagnosed from 1994 to 2010, and investigated the prognostic significance of HPV status and several biomarkers, including p16.

The research paper by Stephen et al investigated the clinical significance of p16 status in various HPV positive and HPV negative head and neck cancers sub sites, including the oropharynx. They selected a pilot cohort of 80 HNSCC cases, which consisted of 10 HPV positive and 10 HPV negative tumours from 4 sites (oral cavity, oropharynx, larynx and hypopharynx). These cases were identified from the Henry Ford Health System (Detroit, USA), and diagnosed in the period of 1986 to 2003. The selection criteria of specific cases were not described.

Finally, the study conducted by Weinberger et al investigated the clinical significance of p16 expression in OPC. The authors identified 107 patients from the Yale-New Haven Hospital database, treated between 1980 and 1999 with primary radiotherapy or surgery and postoperative radiotherapy. They excluded cases of metastatic disease or incomplete therapy.

### 2.3.2.2 HPV and p16 testing

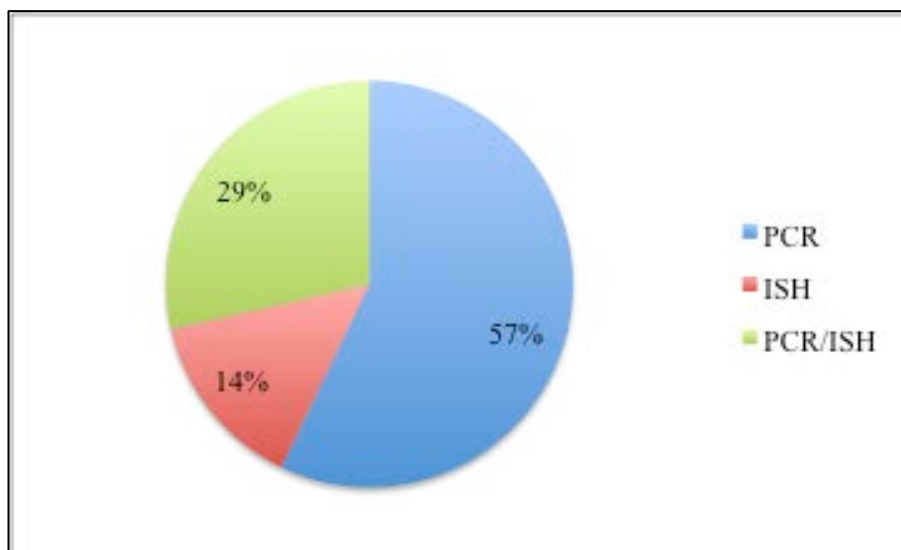


Figure 2.2: HPV testing methods in the included studies

The results in Figure 2.2 demonstrate the majority of the included studies utilised PCR to detect HPV in the tumours. One study (Song et al) used ISH only, while 2 studies (Evans et al and Lewis et al) used both methods. The PCR kits and controls used varied between the studies. However, all the studies that utilised ISH for HPV detection used the Inform HPV III Family 16 probe.

The prevalence of HPV in the included studies ranged from 36-68%, and is summarised in Table 2.7. The average HPV prevalence was 54%. Interestingly the lowest prevalence was found in the South Korean study. The majority of HPV positive cases among the studies were HPV 16 genotype, with an average prevalence of 95%.

Study	HPV Prevalence	HPV 16 Prevalence
Evans et al	50%	97%
Hong et al	57%	95%
Junor et al	Cohort 1 = 41% Cohort 2 = 63%	92% (combined)
Lewis et al	68%	*
Song et al	36%	*
Stephen et al	N/A	N/A
Weinberger et al	61%	*
<b>Average prevalence</b>	<b>54%</b>	<b>95%</b>

*Table 2.7: HPV and HPV16 prevalence between studies. N/A- Stephen et al paper used a selective pilot cohort, not representative of true HPV prevalence. \*Data not provided*

Testing of p16 expression involved immunohistochemistry assays, with the included studies using either the CINtec Histology kit or DAKO EnVision Flex+ system. The percentage staining used to define p16 positivity varied amongst the

research papers (Figure 2.3). However, all the included studies defined positivity as diffuse nuclear and cytoplasmic staining.

The variations in both HPV and p16 testing, including cut-off points, do introduce a significant bias to these results and must be considered when assessing the outcomes of this review.

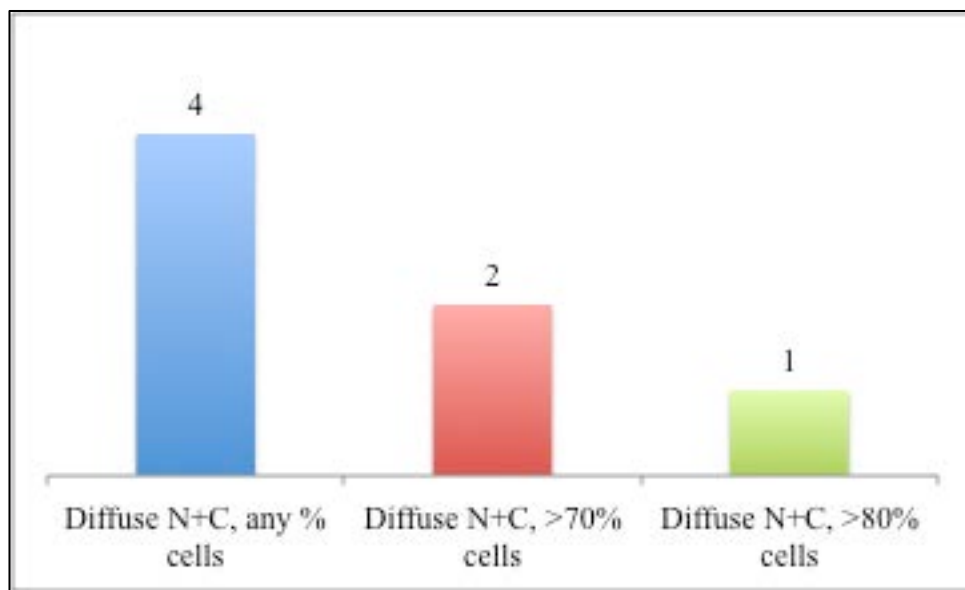


Figure 2.3: The number of studies utilising various p16 staining and percentage patterns. N+C- Nuclear and Cytoplasmic

### 2.3.2.3 Quality assessment scores

Both reviewers independently scored the included papers according to the criteria detailed in Section 2.2.3. These are summarised in Table 2.8. The individual scores in each category are detailed in the Appendix. The quality scores for reviewer 1 (MJ) ranged from 4 to 10, with a mean score of 7.5 (out of 12). Reviewer 2 (NG) assigned scores from 5 to 12, with a mean score 7.75.

<b>Study</b>	<b>Reviewer 1 (MJ) Total score</b>	<b>Reviewer 2 (NG) Total score</b>	<b>Average Total score</b>
Evans et al	10	12	11
Hong et al	9	8	8.5
Junor et al	8	9	8.5
Lewis et al	7	7	7
Song et al	4	5	4.5
Stephen et al	4	5	4.5
Weinberger et al	8	7	7.5
<b>Overall average</b>	<b>7.25</b>	<b>7.75</b>	<b>7.5</b>

*Table 2.8: Quality assessment scores (total out of 12) given by both reviewers for each study*

### 2.3.3 Pooled study demographics

The demographics from each study were extracted from the selected papers and pooled according to the sub-groups of HPV/p16 status. These will be discussed in further detail in the descriptive analysis.

#### 2.3.3.1 Sub-group characteristics

The total number of subjects in each sub-group, sex distribution and average of the mean age are summarised in Table 2.9. The majority of subjects (80%) appeared in the HPV-/p16- and HPV+/p16+ groups, with the discordant groups accounting for a minority of cases (20%). This trend is similar to the collated results seen in Table 1.1. All the sub-groups had a higher proportion of male patients, as seen in previous literature (Section 1.1.3.6). The sex ratio between the HPV-/p16- and HPV+/p16- were similar, approximately 3:1. In comparison the HPV+/p16+ and HPV-/p16+ both had a higher proportion of male subjects, approximately 4:1 and 9:1 respectively.



By collating the mean ages described in each study, an average value was derived in order to compare between the sub-groups. As with the sex distribution, it appears that the HPV-/p16- and HPV+/p16- groups have comparable values of 61 and 60.4 years respectively, with similar mean age ranges. The two other sub-groups, HPV+/p16+ and HPV-/p16+, had a lower average mean age of 55.3 and 52.2 years respectively.

Sub-group	Total number of subjects (%)	Male (%)	Female (%)	Average of mean age in years (range)
HPV-/p16-	471 (35%)	336 (75%)	114 (25%)	61 (57-66.1)
HPV+/p16-	194 (15%)	131 (71%)	54 (29%)	60.4 (57-63.3)
HPV+/p16+	604 (45%)	478 (82%)	102 (18%)	55.3 (54.5-55.7)
HPV-/p16+	61 (5%)	36 (92%)	3 (8%)	52.2 (48.4-55.7)

Table 2.9: Summary characteristics of the HPV/p16 sub-groups

### 2.3.3.2 Smoking history

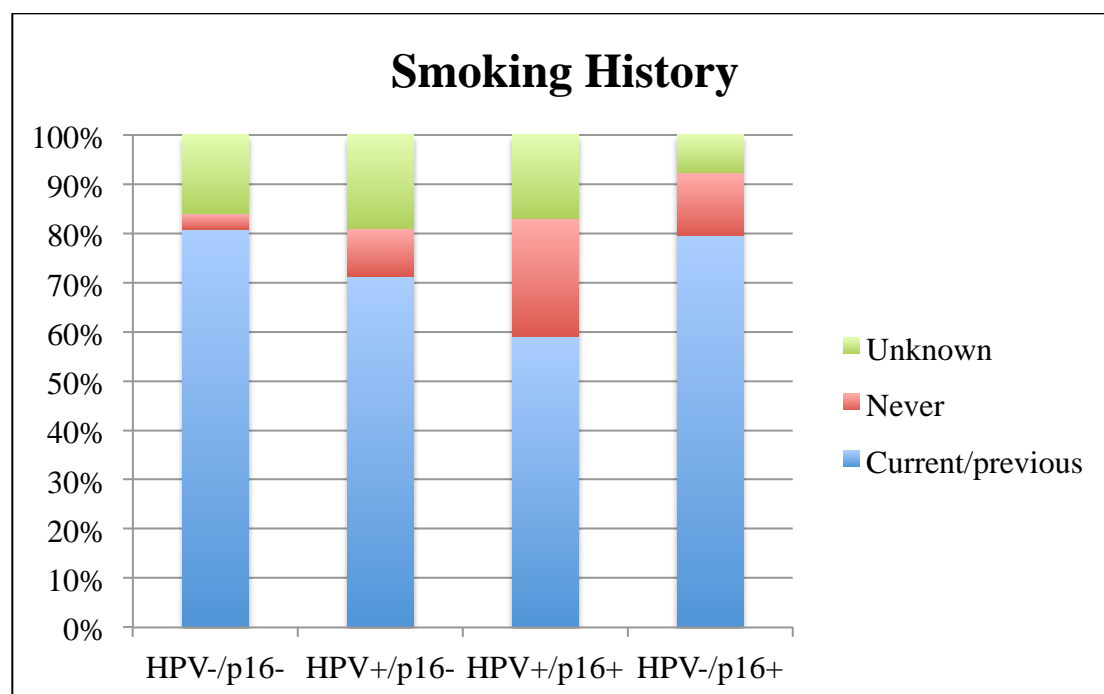


Figure 2.4: Smoking status within each HPV/p16 sub-group. Data collated from Evans et al, Hong et al, Junor et al, Lewis et al, and Weinberger et al

All the studies, apart from Song et al and Stephen et al, provided data on smoking status. In Figure 2.4 the smoking status between the sub-groups are compared. Both the HPV-/p16- and HPV-/p16+ groups were associated with the highest proportion of current/previous smoking history, approximately 80%. The HPV-/p16- group also had the lowest percentage (3%) of non-smokers. The HPV+/p16- group had 71% subjects with a history of smoking. In comparison, the HPV+/p16+ group had the lowest proportion (60%) of subjects with a positive smoking history, and the highest percentage of non-smokers (24%).

### *2.3.3.3 Drinking status*

The drinking status of each sub-group was only adequately described in the study by Junor et al, and is summarised in Figure 2.5. This study provided 2 separate cohorts, and the results have been combined. However, there was no information available for the HPV-/p16+ group. There was also no clear definition of the terms 'excess' and 'social' drinking.

The results show a similar pattern between the HPV-/p16- and HPV+/p16- groups, with 51% and 48% respectively defined as excess drinkers. Social drinkers in these two groups were 32% and 30% respectively. In comparison the HPV+/p16+ group had 23% excess drinkers and 60% social drinkers.

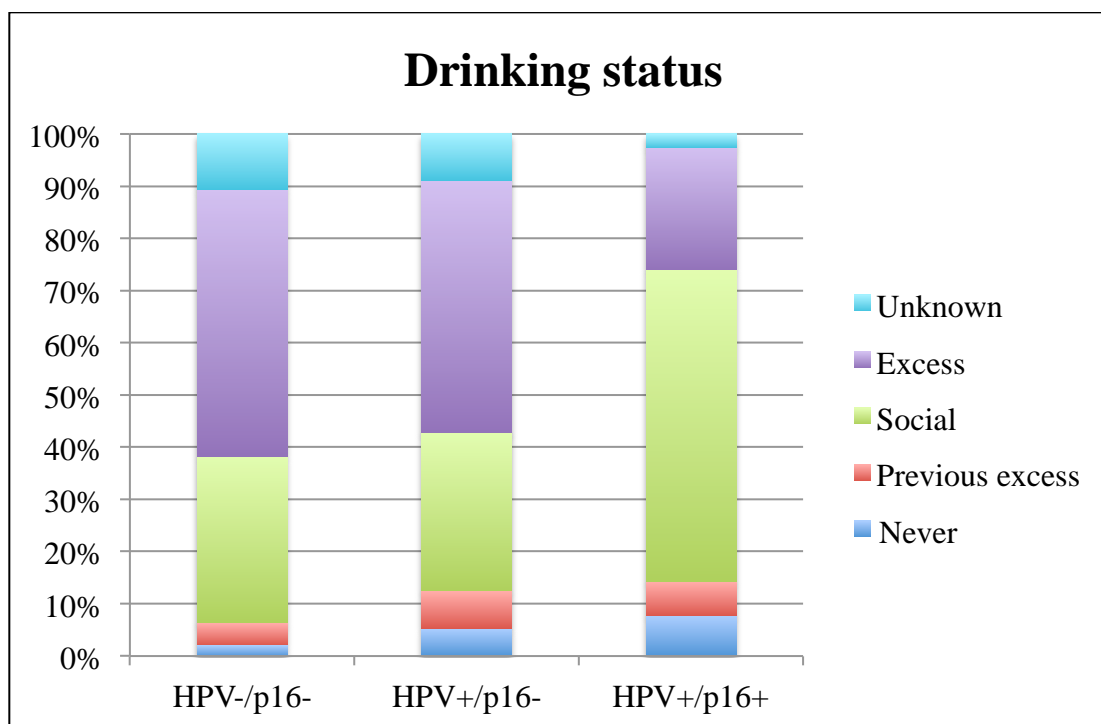
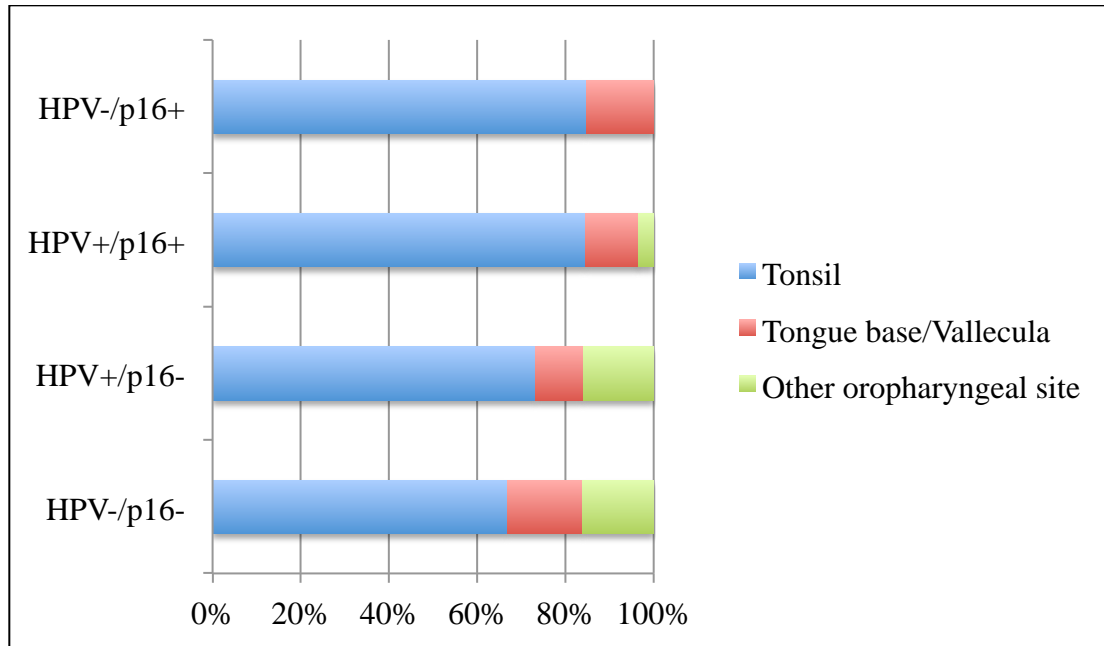


Figure 2.5: Drinking status of the sub-groups extracted from the Junor et al study.  
NB- Data for HPV-/p16+ group was not provided

#### 2.3.3.4 Primary tumour sites

There were 3 studies that provided detailed information on primary tumour sites for each sub-group (Evans et al, Hong et al and Junor et al). The study by Song et al only investigated tonsillar SCC tumours, and so was excluded in the analysis demonstrated in Figure 2.6. The most common primary site of tumours in all sub-groups is the tonsil. There appears to be a higher proportion of tonsillar tumours (85%) in the HPV+/p16+ and HPV-/p16+ sub-groups, with a lower proportion of other oropharyngeal sites (4% and 0% respectively). In contrast the HPV-/p16- and HPV+/p16- show a higher percentage of other oropharyngeal sites, both 16%.



*Figure 2.6: Primary tumour sites combined for each sub-group. NB- other oropharyngeal sites included soft palate, posterior pharyngeal wall, uvula or not otherwise specified. Data collated from Evans et al, Hong et al, and Junor et al*

#### 2.3.3.5 TNM stage

The results in Figure 2.7 summarise the TNM staging for each sub-group. These were collated from Evans et al, Hong et al, Junor et al and Weinberger et al. There appears to be a higher proportion of Stage IV disease among the HPV-/p16+ and HPV+/p16+ groups of 62% and 72% respectively. Comparing the HPV-/p16- and HPV+/p16- groups the percentage of Stage IV disease is 49% and 48% respectively. The proportion of Stage I/II disease in the HPV-/p16- and HPV+/p16- groups is 25% and 24% respectively.

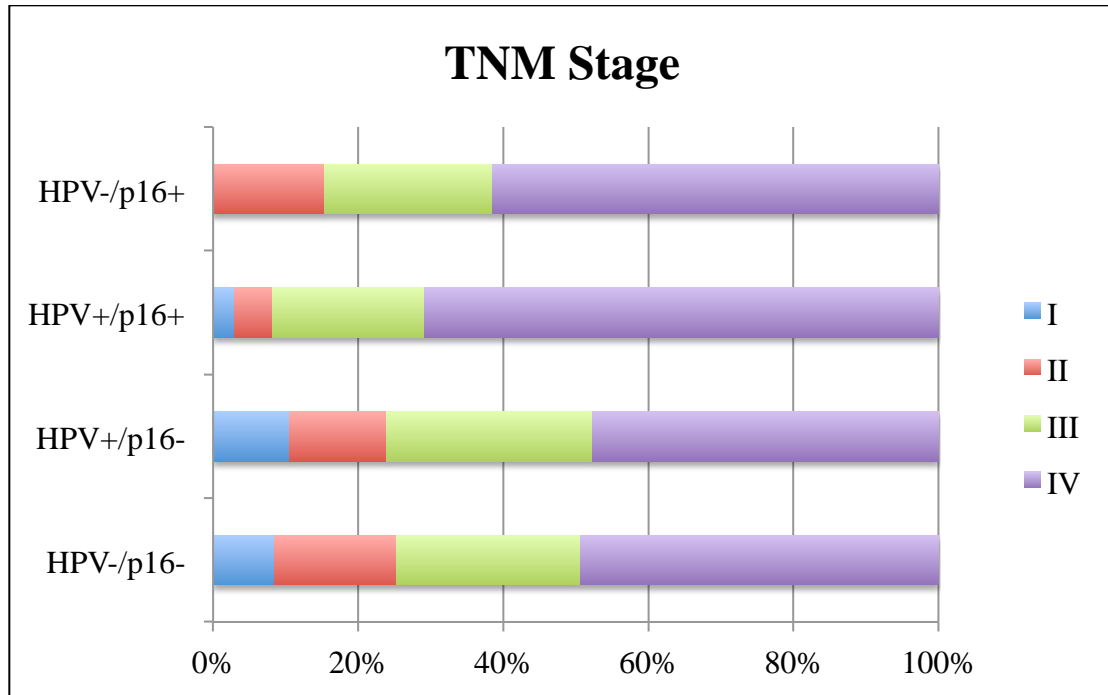


Figure 2.7: TNM staging for each sub-group. Data collated from Evans et al, Hong et al, Junor et al, and Weinberger et al

#### 2.3.3.6 Tumour grade

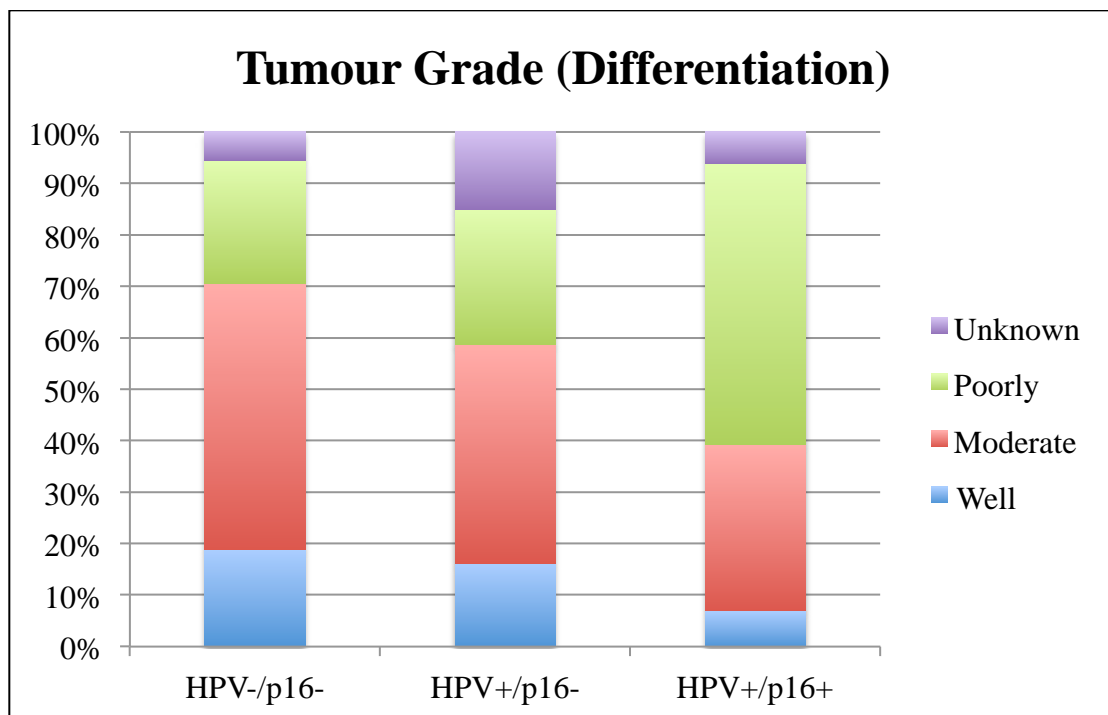


Figure 2.8: Tumour grade summarised according to sub-groups. NB- Insufficient data for HPV-/p16+ sub-group. Data collated from Hong et al, Junor et al, and Weinberger et al

Three studies provided sufficient data to summarise tumour grade (Figure 2.8) between sub-groups (Hong et al, Junor et al and Weinberger et al). Both the HPV-/p16- and HPV+/p16- groups demonstrated a higher proportion of well/moderately-differentiated tumours compared to HPV+/p16+ group, approximately 71%, 59% and 39% respectively. In contrast the majority (54%) of HPV+/p16+ tumours were poorly differentiated, compared to HPV-/p16- (24%) and HPV+/p16- (26%).

### 2.3.3.7 Primary treatment

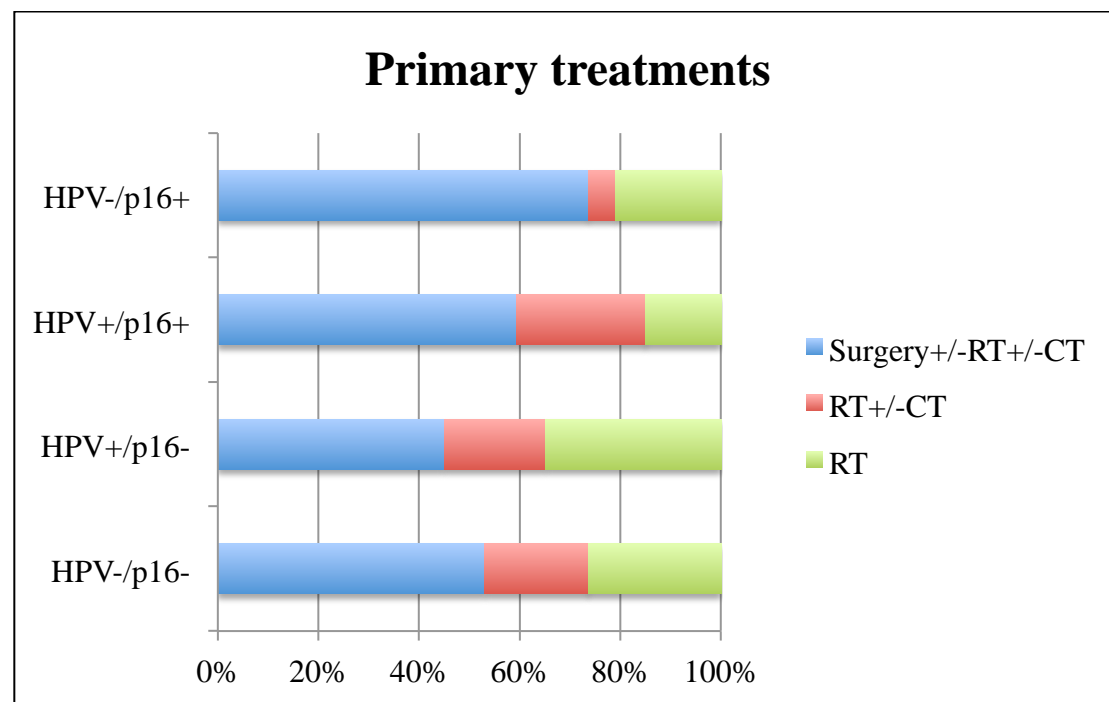


Figure 2.9: Differences in primary treatments between sub-groups. RT- radiotherapy, CT- chemotherapy. Data collated from Evans et al, Hong et al, Junor et al, Lewis et al, and Weinberger et al

All the included studies provided information on primary treatments, apart from Song et al and Stephen et al. Treatment categories between studies did vary, and therefore treatments have been grouped into three general groups, and these are

summarised in Figure 2.9. The HPV-/p16+ (74%) and HPV+/p16+ (59%) groups had a higher percentage treated by surgery +/- radiotherapy +/- chemotherapy, compared to HPV-/p16- (53%) and HPV+/p16- (45%). In contrast a higher proportion of cases in the HPV+/p16- (35%) and HPV-/p16- (26%) groups were treated with radiotherapy alone, compared to the HPV+/p16+ (15%) and HPV-/p16+ groups (21%).

### **2.3.4 Pooled prognostic outcomes**

After discussion with the biostatistician a meta-analysis was deemed inappropriate due to the limited number of included studies and lack of adequate survival data (i.e. hazard ratios and confidence intervals). The prognostic outcomes in the selected studies were mainly presented in Kaplan-Meier survival plots. The following data have been extracted from these graphs in order to represent pooled prognostic outcomes, but statistical testing and significance would not be valid in this case.

#### *2.3.4.1 Disease-free survival*

The results in Tables 2.10 and 2.11 summarise the 3 and 5-year disease-free survival (DFS) rates respectively, produced by data extracted from Kaplan-Meier plots. The average DFS rates are graphically represented in Figure 2.10. The lowest DFS rate is shown in the HPV-/p16- group, with the HPV+/p16- group demonstrating comparable rates. In comparison the highest DFS rate is observed in the HPV+/p16+ group, with the HPV-/p16+ group also showing similar DFS rates.

Study	3 year DFS			
	HPV-/p16-	HPV+/p16-	HPV+/p16+	HPV-/p16+
Lewis et al	0.3	*	0.8	0.7
Song et al	0.35	0.5	0.82	0.72
Weinberger et al	0.3	0.35	0.76	*
<b>Average DFS</b>	<b>0.32</b>	<b>0.43</b>	<b>0.79</b>	<b>0.71</b>

Table 2.10: The 3 year DFS data extracted from Kaplan-Meier plots. \*Data not provided

Study	5 year DFS			
	HPV-/p16-	HPV+/p16-	HPV+/p16+	HPV-/p16+
Lewis et al	0.25	*	0.75	0.7
Song et al	0.35	0.5	0.82	0.72
Weinberger et al	0.15	0.13	0.76	*
<b>Average DFS</b>	<b>0.25</b>	<b>0.32</b>	<b>0.78</b>	<b>0.71</b>

Table 2.11: The 5 year DFS data extracted from Kaplan-Meier plots. \*Data not provided

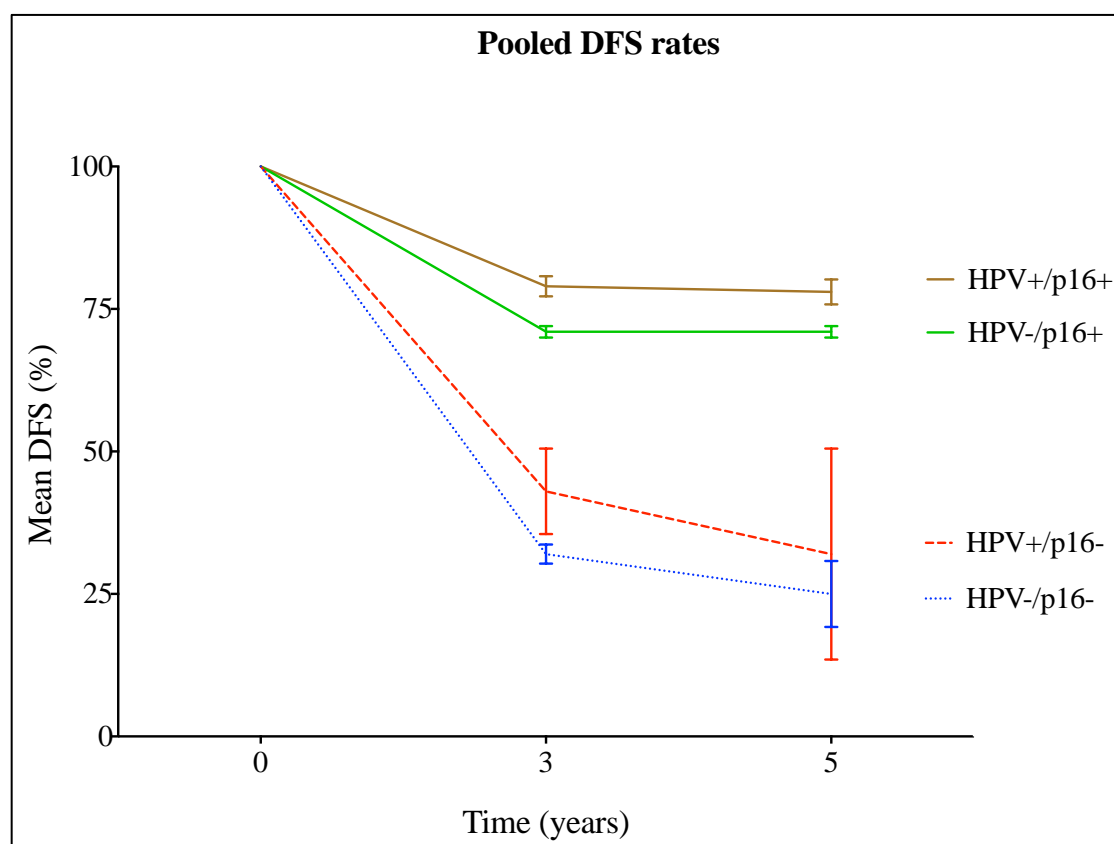


Figure 2.10: Percentage mean DFS data pooled from the included studies, with standard error of means



### 2.3.4.2 Disease-specific survival

Only 2 studies provided data for disease-specific survival (DSS), as summarised in Tables 2.12 and 2.13. These demonstrate the lowest DSS rates in the HPV-/p16- group, and the highest in the HPV+/p16+ group. Data for the other discordant groups were only provided in the separate papers, and therefore an average value could not be obtained. In the study by Hong et al the HPV+/p16- group had the lowest 3 and 5-year DSS of 61% and 58% respectively. The study by Lewis et al demonstrated similar DSS rates between the HPV-/p16+ and HPV+/p16+ groups.

Study	3 year DSS			
	HPV-/p16-	HPV+/p16-	HPV+/p16+	HPV-/p16+
Hong et al	0.65	0.61	0.87	*
Lewis et al	0.5	*	0.85	0.85
<b>Average DSS</b>	<b>0.58</b>	<b>N/A</b>	<b>0.86</b>	<b>N/A</b>

*Table 2.12: The 3 year DSS data extracted from Kaplan-Meier plots. \*Data not provided*

Study	5 year DSS			
	HPV-/p16-	HPV+/p16-	HPV+/p16+	HPV-/p16+
Hong et al	0.63	0.58	0.75	*
Lewis et al	0.5	*	0.75	0.7
<b>Average DSS</b>	<b>0.57</b>	<b>N/A</b>	<b>0.83</b>	<b>N/A</b>

*Table 2.13: The 5 year overall DSS extracted from Kaplan-Meier plots. \*Data not provided*

### 2.3.4.3 Overall survival

The overall survival rates were extracted from Kaplan-Meier plots and summarised in Tables 2.14 and 2.15. Only the study by Stephen et al did not provide

data in this format. The average 3 and 5-year overall survival rates were lowest in the HPV-/p16- group, with the highest survival demonstrated in the HPV+/p16+ cohort. The discordant groups demonstrated survival rates between these two groups, with the HPV-/p16+ group revealing better overall survival rates compared to HPV+/p16- group. The pattern of overall survival between these groups is demonstrated in Figure 2.11.

Study	3 year Overall Survival			
	HPV-/p16-	HPV+/p16-	HPV+/p16+	HPV-/p16+
Evans et al	0.32	0.66	0.82	0.5
Hong et al	0.55	0.55	0.85	*
Junor et al (Cohort 1)	0.5	0.3	0.88	*
Junor et al (Cohort 2)	0.35	0.75	0.9	*
Lewis et al	0.3	*	0.8	0.8
Song et al	0.5	0.5	0.95	0.7
Weinberger et al	0.35	0.5	0.79	0.5
<b>Average OS</b>	<b>0.41</b>	<b>0.54</b>	<b>0.86</b>	<b>0.67</b>

Table 2.14: The 3 year overall survival data extracted from Kaplan-Meier plots.

\*Data not provided

Study	5 year Overall Survival			
	HPV-/p16-	HPV+/p16-	HPV+/p16+	HPV-/p16+
Evans et al	0.24	0.5	0.75	0.5
Hong et al	0.45	0.47	0.75	*
Junor et al (Cohort 1)	0.4	0.18	0.82	*
Junor et al (Cohort 2)	0.35	0.7	0.88	*
Lewis et al	0.25	*	0.75	0.65
Song et al	0.32	0.5	0.95	0.58
Weinberger et al	0.2	0.18	0.79	0.5
<b>Average OS</b>	<b>0.32</b>	<b>0.42</b>	<b>0.81</b>	<b>0.58</b>

Table 2.15: The 5 year overall survival data extracted from Kaplan-Meier plots.

\*Data not provided

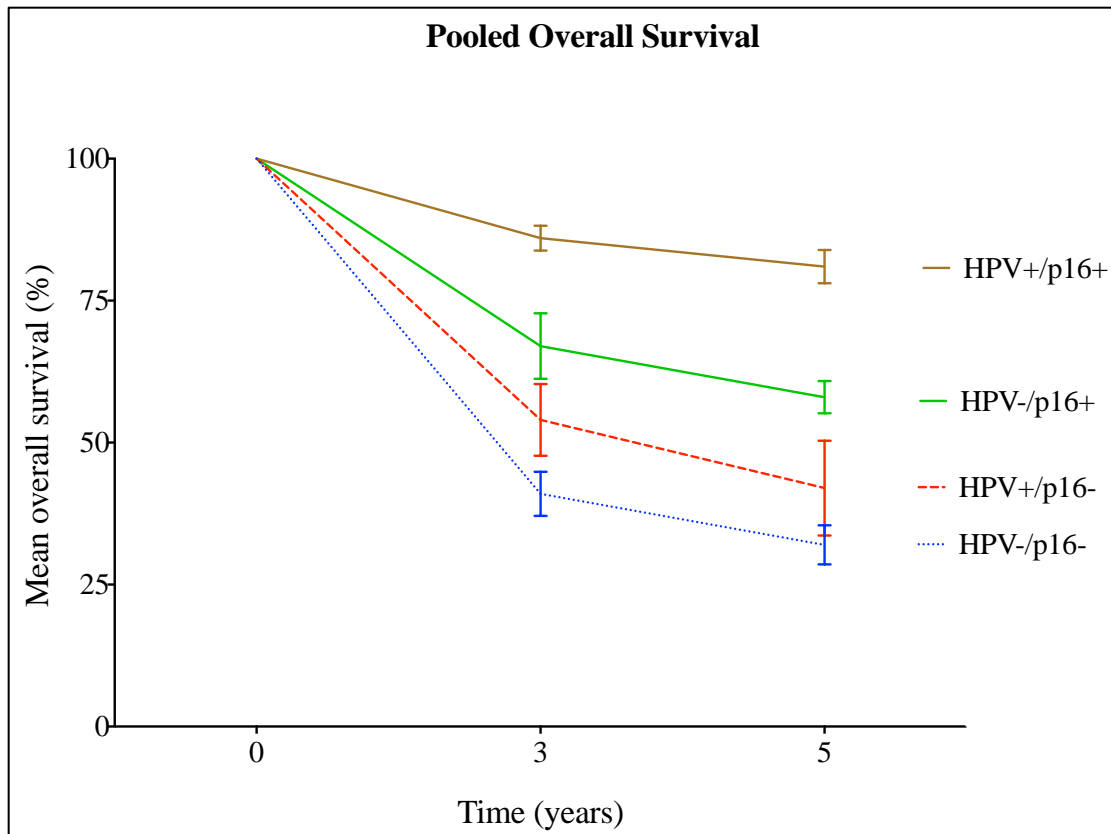


Figure 2.11: Percentage mean overall survival data pooled from the included studies, with standard error of means

#### 2.3.4.4 Hazard ratios

In total 5 studies reported hazard ratios (HR) of death from any cause. These are summarised in Table 2.16. The reference group used for each study varied, with three papers choosing the HPV-/p16- group and the other two studies referencing against the HPV+/p16+ group. The diversity of the presented data prevented a meta-analysis. Comparing between studies, the best prognostic cohort appeared to be HPV+/p16+, with the HPV-/p16- the worst. The HPV+/p16- group showed similar HR compared to HPV-/p16-. In contrast the HPV-/p16+ group had lower HR values compared to HPV-/p16-.

Study	Hazard Ratios (Confidence Intervals)			
	HPV-/p16-	HPV+/p16-	HPV+/p16+	HPV-/p16+
Hong et al	Reference group	1.04 (0.77-1.41)	0.33 (0.25-0.45)	*
Lewis et al	Reference group	*	0.21 (0.13-0.36)	0.26 (0.12-0.62)
Weinberger et al	Reference group	0.80 (0.40-1.80)	0.19 (0.10-0.70)	*
<b>AVERAGE</b>	<b>Reference</b>	<b>0.92</b>	<b>0.24</b>	<b>N/A</b>
Song et al	5.34 (1.11-25.81)	3.05 (0.27-33.89)	Reference group	3.23 (0.65-16.15)
Stephen et al	10.30 (1.20-88.33)	11.44 (0.69-191.0)	Reference group	6.98 (0.61-79.47)
<b>AVERAGE</b>	<b>7.82</b>	<b>7.23</b>	<b>Reference</b>	<b>5.12</b>

Table 2.16: Sub-group summary of hazard ratios of death from any cause. \*Data not provided

## 2.4 Discussion

There has been substantial research and increasing evidence in recent years of the role of HPV in HNSCC pathogenesis, particularly oropharyngeal cancers. Various studies indicate HPV positive HNSCC patients show significantly improved survival outcomes compared to HPV negative cases. Utilising HPV status as part of risk stratification and prognostic models requires a robust and accurate system of diagnosis. There are a number of diagnostic tests for detecting HPV, including p16 expression, which were discussed in Chapter 1. While HPV and p16 status correlate in the majority of cases, the significance of the 2 discordant groups (HPV+/p16- and HPV-/p16+) remains unclear. This is the first systematic review to investigate the prognostic outcomes of OPC based on the HPV/p16 combination.

### **2.4.1 True positive vs. True negative**

The majority of OPC cases in previous studies showed a strong correlation between HPV and p16 status, as summarised in Table 1.1. The studies included in this review found a similar pattern, with a total of 80% of cases either HPV+/p16+ (true positive) or HPV-/p16- (true negative).

#### *2.4.1.1 Demographics*

Comparing between the true positive and true negative cohorts the results of this review demonstrate clear differences with the group characteristics and prognostic outcomes, as seen in previous studies. The sex ratio in the HPV-/p16- cohort was 3:1 (male: female) compared to 4:1 with HPV+/p16+. The higher proportion of male subjects in HNSCC is well established (Langevin et al., 2011). The study of OPC cases by Ang et al showed a similar increased proportion of male subjects in the HPV positive cohort compared to HPV negative (86.4% vs. 79.5% respectively), but this was not statistically significant (Ang et al., 2010). We also found a difference in mean age at diagnosis of 61 years (true negatives) and 53 years (true positives). Previous studies have demonstrated statistically significant differences in the average ages between HPV positive and HPV negative cases (Ang et al., 2010, Chaturvedi et al., 2011).

The typical risk factors associated with HNSCC are smoking and alcohol consumption, and these showed variations between the two cohorts. In the HPV-/p16- group 81% had a positive smoking history, compared to 59% in the HPV+/p16+

group. However, excluding the cases with an unknown smoking history the figures are 96% and 71% respectively. The RTOG 0129 data (Ang et al., 2010) of OPC cases reported on the median years of tobacco exposure, with 36.5 years in the HPV negative cohort compared to 12.2 years in HPV positive cases ( $p < 0.001$ ). In this review we also found a discrepancy in alcohol exposure between the true HPV positive and negative cohorts, albeit based on only one study by Junor et al. On average 51% of HPV-/p16- cases were classified as excessive drinkers, compared with 23% of HPV+/p16+ cases. Exposure to both alcohol and smoking is thought to play a synergistic effect on the risk of cancer (Castellsague et al., 2004).

Within the oropharynx several anatomical sub-sites exist, and our data illustrates a difference between the HPV-/p16- and HPV+/p16+ groups. In total 84% HPV-/p16- cases were either tonsil or base of tongue tumours, compared to 96% of HPV+/p16+. The remaining cases were located in other oropharyngeal sites including uvula, posterior pharyngeal wall and soft palate. In a Swedish study of OPC cases the prevalence of HPV at the different sub-sites were analysed (Attner, 2013). The majority of tonsillar and base of tongue SCC were HPV positive (79% and 75% respectively), whereas 25% of other OPC sites were positive. In another Swedish study focusing on OPC cases other than tonsil or base of tongue sites, 69% cases were HPV-/p16- (Marklund et al., 2012). Furthermore the authors found that HPV or p16 status in these sub-sites showed no prognostic significance.

There is evidence that HPV-related oropharyngeal cancers present at a more advanced disease stage, with a lower T-stage and higher N-stage (Elrefaey et al., 2014, Psyrris et al., 2011). This review found 25% HPV-/p16- cohort were TNM stage

I/II, compared to 8% in the HPV+/p16+ group. The majority of HPV+/p16+ cases were TNM stage IV compared to HPV-/p16- (71% and 49% respectively). In a recent UK study of OPC cases there was a statistically significant difference in TNM staging between the HPV negative and HPV positive cohorts, with 51.3% and 80.3% respectively classified as stage IV (Ward et al., 2014). Our review also demonstrated that HPV+/p16+ cases had a greater proportion of poorly differentiated tumours compared to HPV-/p16- (54% and 24% respectively). Other studies have shown HPV positive OPC associated with poorly differentiated tumours (Gillison et al., 2000).

In summary our review confirms results from previous studies regarding the differences in demographics and risk profile between true positive and negative OPC. The typical true positive patient would be male, younger, social drinker, and less likely to be a smoker. The true positive tumours are more likely to be tonsil or base of tongue, diagnosed at a more advanced stage, and poorly differentiated. Whereas the true negative patient would likely be an older male, with a positive smoking history and an excessive drinker with a greater chance of the primary tumour located in other oropharyngeal sites, and presenting at an earlier stage with more a favourable tumour grade.

### *2.4.1.2 Prognostic outcomes*

The results of this systematic review concur with numerous studies and reviews on the subject of HPV positivity and improved prognostic profiles. The 3 and 5-year DFS rates for HPV-/p16- cases were 32% and 25% respectively. In contrast the figures for HPV+/p16+ are far more favourable at 79% and 78% respectively.

Similarly the 3 and 5-year DSS rates for HPV-/p16- were 58% and 57%, compared to HPV+/p16+ of 86% and 83%. The same pattern is observed for 3 and 5-year overall survival rates, with HPV-/p16- values of 41% and 32%, and HPV+/p16+ of 86% and 81%.

Five studies in this systematic review provided data on HR of death, with all showing a significantly better HR for the HPV+/p16+ group. Referencing against HPV-/p16- there was on average a 76% reduced risk of death for the HPV+/p16+ group (average HR 0.24). There were 2 papers that referenced against the HPV+/p16+ group, with the average HR for HPV-/p16- of 7.82. In comparison the systematic review by Ragin et al demonstrated a 28% (HR 0.72) reduced risk of death for HPV positive OPC cases compared to HPV negative (Ragin et al., 2007b). In the meta-analysis by Dayyani et al the HR of HPV positive OPC was 0.40 (Dayyani et al., 2010), which was statistically significant ( $p < 0.0001$ ). Another review article found HPV positive OPC had a pooled HR of 0.47 (O'Rorke et al., 2012).

The demographic differences described between the two main groups of OPC are likely to influence the prognostic outcomes. The younger HPV+/p16+ cohort has less association to smoking and alcohol, both of which are classified as group 1 carcinogens (IARC, 2014). There is evidence that HPV negative tumours are more likely to harbour critical mutations of genes related to cell survival, such as p53. One review article (Dayyani et al., 2010) reported p53 mutations in HPV positive HNSCC were statistically less likely compared to HPV negative tumours (Adjusted OR = 0.21,  $p = 0.015$ ). Besides the differences in risk factors between these groups, the younger



HPV+/p16+ patient is less likely to present with multiple co-morbidities, which can also influence treatment options and survival outcomes.

However, one paradox of these cancers is that HPV positive OPC typically present at a more advanced stage and with poorly differentiated tumours compared to HPV negative cases. This pattern was also demonstrated in our review in the HPV+/p16+ cohort. Logically such tumours would be expected to have poorer response to treatment and worse prognosis, but the data contradicts this notion. Therefore other undefined factors are likely to play a role in the natural history of HPV positive disease. Theoretically the link between smoking and alcohol with regards to increased risk of mutations may render the HPV negative tumours cells more molecularly unstable, and thus less likely to respond well to treatments. Moreover, the overexpression of p16 in the majority of HPV positive tumours is likely to influence the prognostic outcomes. The role of p16 in normal cell function was described in Section 1.2.3.4. Previous studies have demonstrated p16 as an independent positive prognostic indicator in OPC (Weinberger et al., 2004, Fischer et al., 2010).

### **2.4.2 Relevance of discordant groups**

The primary aim of this systematic review was to establish the prognostic significance of the discordant groups, HPV+/p16- and HPV-/p16+. The results have demonstrated an interesting pattern, with each discordant group appearing to correlate closely with the true negative and positive cohorts respectively.

### 2.4.2.1 HPV+/p16- vs. True negative

The descriptive analysis has shown that the HPV+/p16- discordant group share similar characteristics to the HPV-/p16- group (true negatives). The HPV+/p16- cohort had a sex ratio of 2.45:1 (M: F), compared to 3:1 in the true negative group. The average mean ages were almost identical at 60.4 years and 61 years respectively.

On average 71% of the HPV+/p16- group had a positive smoking history, compared to 81% in the HPV-/p16- cohort. Based on the study by Junor et al alcohol intake was almost identical between the two groups, with approximately 48% of the HPV+/p16- group classified as excess drinkers and 30% social drinkers, and for HPV-/p16- these were 51% and 32% respectively. The proportions in site of primary tumour were the same in both groups, with 84% tonsil or base of tongue and the remaining in other oropharyngeal sites.

Patterns in TNM staging were almost identical; 24% HPV+/p16- were stage I/II, with 25% for the true negative cohort. Similarly 48% and 49% were TNM stage IV respectively. While 59% of HPV+/p16- tumours were well or moderately differentiated, compared to 71% for true negative tumours. In comparison 26% HPV+/p16- tumours were poorly differentiated, which was similar to the true negatives (24%).

As well as demographic profiles, the two groups also share comparable poorer prognostic outcomes. The 3 and 5-year DFS rates for HPV+/p16- were 0.43 and 0.32 respectively, and 0.32 and 0.25 for the true negative cohort. The average 3 and 5-year

overall survival rates for HPV+/p16- were 0.54 and 0.42, compared to 0.41 and 0.32 for HPV-/p16-. The average HR for death for HPV+/p16- when referenced against HPV-/p16- was 0.92. Similarly when referenced against HPV+/p16+ the average HR for HPV+/p16- was 7.23, compared to 7.82 for HPV-/p16-.

In summary the demographic profiles are comparable between HPV+/p16- and the true negative cohort. The prognostic outcomes for HPV+/p16- appear to correlate closely to the HPV-/p16- group, although the latter appear to have the worst prognosis of all the sub-groups.

According to these results the HPV status of tumours may not necessarily indicate a better prognosis, particularly in the sub-group of p16 negative cases. As with the true negative cohort, the prognosis of the HPV+/p16- group maybe influenced by a more unfavourable demographic and risk factor profile. The negative status of p16 for these two groups can also contribute to the poorer outcomes observed. The lack of p16 overexpression in the HPV+/p16- discordant group can be due to three reasons. Firstly, previous studies have reported p16 gene alterations or deletions in a significant proportion of HNC (Liggett et al., 1998). Thus despite a HPV infection the overexpression of p16 would be prevented in such tumours. Furthermore loss of normal p16 function could render the host cells more susceptible to tumourgenesis. Secondly, the HPV+/p16- group may represent true negative tumours with false positive HPV testing, particularly with the highly sensitive PCR method. False positive testing can occur in contaminated samples or HPV infection unrelated to tumour pathogenesis (i.e. non-integrated HPV genome). Therefore it is unclear if the HPV+/p16- cohort represents a true discordant group, although survival

outcomes do appear somewhat more favourable compared to HPV-/p16-. Finally, categorising these groups relies on various laboratory tests and cut-off points, which do differ between the included studies. Also without a clear and uniform definition for each subgroup there is likely to be overlap among the cohorts. This can impact on the results of the discordant groups as well as the true positive and negative subgroups.

### *2.4.2.2 HPV-/p16+ vs. True positive*

The HPV-/p16+ group demonstrated the highest sex ratio difference of approximately 9:1 male to female, followed by the true positive group at 4:1. Both these groups had similar mean ages, 52.2 years for HPV-/p16+ compared to 55.3 years for HPV+/p16+. Positive smoking history was observed in 79% of the discordant HPV-/p16+ group, compared to 59% for HPV+/p16+.

There were similarities between the primary tumour sites; 100% of HPV-/p16+ group were either tonsil or base of tongue tumours, compared to 96% for the true positive cohort. Similarly the pattern of TNM staging showed 15% HPV-/p16+ classed as stage I/II and 8% of HPV+/p16+, while the majority in both groups were stage IV (62% and 71% respectively). Data was unavailable to compare tumour grades.

Prognostically these groups also appeared to correlate. While the true positives demonstrated the best outcomes for all parameters, the HPV-/p16+ group also had relatively better prognosis compared to the HPV-/p16- and HPV+/p16- cohorts. The 3 and 5-year DFS rates for HPV-/p16+ were both 0.71, while the true positive group

were 0.79 and 0.78 respectively. Whereas the overall survival rates were 0.67 and 0.58 respectively for the discordant group, the true positives were 0.86 and 0.81. The study by Lewis et al provided HR values of 0.26 and 0.21 for HPV-/p16+ and HPV+/p16+ respectively, when referenced against HPV-/p16-. Two other studies provided HR values referenced against HPV+/p16+, with a combined average HR of 5.12 for HPV-/p16+.

It appears that the discordant HPV-/p16+ group does share similar demographic and prognostic profiles compared to the true positive cohort. Both groups appear in a younger population, are strongly associated with tonsil or base of tongue tumours, and present at a more advanced disease stage. However, while prognosis in HPV-/p16+ appears more favourable in comparison to HPV-/p16- and HPV+/p16-, there is still a difference in relation to the true positives. This difference could be explained partly by the higher rate of smokers in the HPV-/p16+ cohort. Several possibilities exist regarding the nature of the discordant HPV-/p16+ group. This cohort may represent HPV+/p16+ tumours with a false negative HPV test, particularly if low numbers of HPV particles exist in the tumour sample. Also the HPV tests utilised by the included studies may have missed other HPV types not detected by conventional PCR or ISH methods. Alternatively p16 overexpression may not be driven by HPV infection but by another unknown variable, such as dysfunction in the pRb pathway leading to loss of p16 feedback control. Like HPV+/p16- it is unclear if HPV-/p16+ tumours represent a true discordant group with specific biological qualities.

### 2.4.3 Prognostic models

This systematic review has highlighted the varying patterns of 4 sub-groups of oropharyngeal cancers according to HPV and p16 status. The HPV+/p16+ patients clearly have more favourable demographics and prognostic outcomes, and the HPV-/p16- are related to poorer demographic and risk factor profiles and associated with the worst prognosis. The two discordant groups have demonstrated similar profiles related to the true positive and negative cohorts respectively, with HPV-/p16+ patients revealing better prognostic outcomes compared to HPV+/p16-. Therefore the p16 status of OPC tumours appears more likely to predict better prognosis than HPV status alone, and adds weight to the argument of utilising p16 testing as a surrogate marker of HPV-related cancers (El-Naggar et al., 2012).

Prognostic models of oropharyngeal cancers can be potentially complex, particularly if focused on the multiple parameters for individual patients. The ultimate model would be applicable to the majority of patients and highly accurate. However, our results indicate that HPV status maybe missing sub-groups of patients with varying survival outcomes. For example, the model proposed by Ang et al (Figure 1.6) initially separates tumours according to HPV status. Therefore the HPV-/p16+ group would be classed as intermediate or high risk, depending on smoking history and T-stage. Similarly the HPV+/p16- would be considered low or intermediate risk according to smoking history and N-stage. The accurate classification of these sub-groups can potentially improve disease outcomes when considering de-escalation or more aggressive treatment options according to risk stratification.

Considering the substantial evidence of improved outcomes in HPV-related cancers, it is only a matter of time before HPV status is universally included in disease staging and risk stratification. Therefore accurate testing is required to identify HPV relevant disease. This can include combining p16 testing with PCR and ISH. Previously there have been algorithms proposed for HNSCC HPV testing, which claim 100% sensitivity and specificity by utilising p16 IHC and PCR (Smeets et al., 2007). Future utilisation of accurate testing algorithms will depend on costs and resource availability.

### **2.4.4 Limitations of systematic review**

In this review we aimed to ensure our searches and selection of relevant studies was comprehensive. However, the accuracy of such searches is limited by the quality of the databases themselves and the search terms used. For example, we found most studies abbreviated oropharyngeal cancer as OPSCC, while a minority abbreviated as OSCC. This could impact on the search results. We did ensure a more precise search strategy by utilising two independent reviewers. The inter-rater reliability scores for title and abstract selection were rated as moderate (0.498) and substantial (0.794) respectively, according to the Kappa score. We intended to include all studies irrespective of language. However, two studies from China and Japan could not be obtained. Only one study was included from Asia.

We found the quality of the included studies was variable, with an average range from 4.5 to 11 (out of 12). The included studies were all retrospective cohorts, which are level IIb evidence. The effect of each study on the overall results can

normally be assessed by sensitivity analysis. However, due to the limited data and number of included studies a formal meta-analysis was not possible. We were also unable to assess the statistical significance of our results.

Another limitation in this review is the variation in p16 and HPV testing among the studies. There was no consensus on defining p16 positivity, with a discrepancy in staining cut-off points. Also while some studies utilised more than one pathologist for p16 scoring others did not mention this. Furthermore, different HPV testing methods were used in each study. Therefore, we could not assess the impact of these laboratory tests on the overall results. Ideally a sensitivity analysis would have been utilised if a meta-analysis were possible, in order to assess the impact of different study variables on the outcome of the review.



## **Chapter 3: Drug Re-purposing**

### 3.1 Introduction

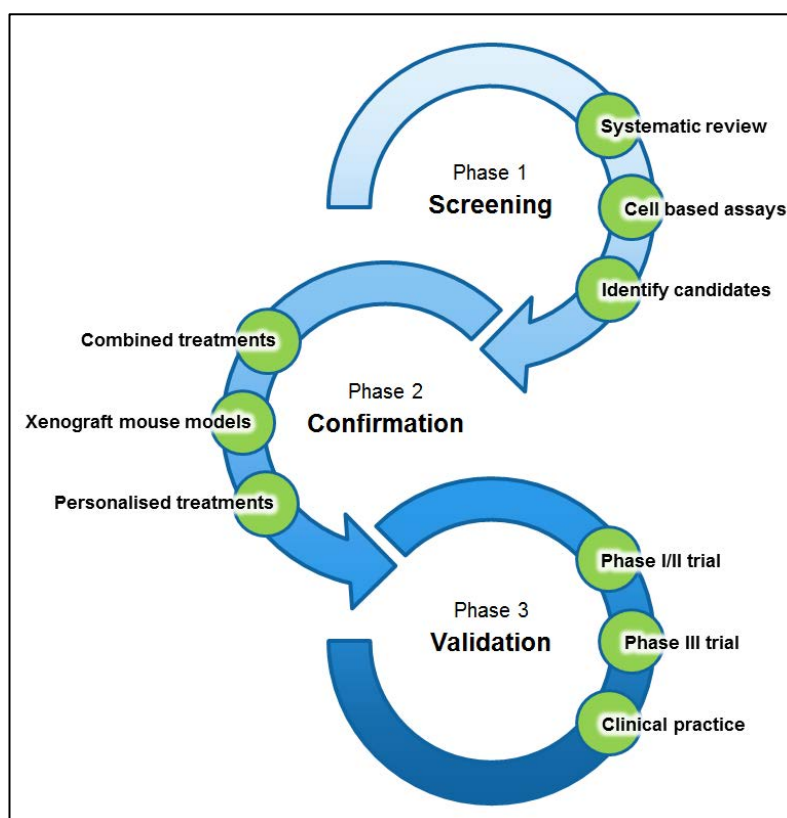
Drug re-purposing has the potential to identify novel therapies for various conditions, including cancers. Cancer treatment, such as chemotherapy, is usually associated with significant side effects. These include nausea and vomiting, bone marrow suppression, loss of hair and mucositis . However, novel therapies using drug re-purposing may offer target drugs with a more favourable side effects profile.

Various drug re-purposing methods exist, such as serendipitous discoveries with current drugs or computational methods to predict new drug targets. In another systematic method high-throughput screening (HTS) can be utilised by automated cell-based assays and drug libraries. This technique has been employed, for instance, in one study to identify potential drug targets for treating thyroid cancers (Zhang et al., 2012). The authors performed HTS with a Luciferase-coupled ATP quantification assay using TPC-1 (human papillary thyroid cancer cell line) and used a publically accessible drug library consisting of over 2800 small-molecule compounds. They identified 2 drug targets (Bortezomib and Ouabin) possessing both high efficacy,  $IC_{50}$  (half maximal inhibitory concentration) values within the nanomolar range and within clinically achievable drug serum concentration. These drug targets were further tested with various thyroid cancer cell lines using proliferation, caspase and flow cytometry assays. Both agents demonstrated antiproliferative effects across all the selected thyroid cancer cell lines, as well as increasing apoptotic activity compared to vehicle control. Furthermore Ouabin showed evidence of cell cycle arrest (increased G2/M phase) during flow cytometry analysis.

Similarly a systematic approach to drug re-purposing has been utilised for the Accelerated Translation of therapeutic agents and Emerging Devices in Head and Neck and Thyroid cancer). The aims of Accelerated Translation are to rapidly increase available therapies in HNC management, and develop personalised treatments (Mehanna, 2014). The stages of Accelerated Translation are screening, confirmation and validation (Figure 3.1). Initial Phase 1 screening involves a systematic review of numerous sources to identify suitable HNC cell lines and both collaborative and commercial drug libraries. Next a HTS cell-based proliferation assay is used to identify potential drug candidates. Further testing involves a validating proliferation assay, flow cytometry, migration and clonogenic assays. In Phase 2 drug combinations are tested for the previously identified drugs, and also conventional treatments already in clinical practice for the disease are included. Drug targets that are identified with therapeutic potential are then tested in appropriate mouse models before exploring personalised treatments. Finally, Phase 3 involves validation of drug targets in clinical trials prior to introduction into clinical practice.

The aim of the current study was to assess 3 drug targets that emerged from the primary screen; INH001, INH002 and INH003. These drugs were identified from the initial HTS and appeared to be effective in both our selected HNC cell lines, and this will be discussed in further detail. Several cell-based assays were utilised to examine the effects of these drug targets against the two HNC cell lines.

INH001 belongs to the group of water-soluble vitamins. INH002 is an anti-hypertensive drug with alpha-adrenergic agonist activity. INH003 is an anti-helminthic drug effective against tapeworm infections.



*Figure 3.1: Illustration of the Accelerated platform, which includes Phase 1 (drug target screening), Phase 2 (target drug confirmation) and Phase 3 (validation in clinical trials) (Mehanna, 2014)*

## 3.2 Materials and Methods

All preparations and experiments were performed in sterile conditions using a HERAsafe Class II biological safety cabinet (Thermo Scientific- S/N 40927282). Unless stated, incubations during experiments were at 37°C and 5% CO<sub>2</sub>.

### 3.2.1 Cell lines

### 3.2.1.1 Cell line characteristics

Candidate HNC cell lines were selected from commercially available sources, where possible. Ideal cells required the ability to grow a monolayer in culture, and were also chosen according to HPV status.

SCC040 (UPCI-SCC-040) is a HPV negative cell line, purchased from The Leibniz Institute DSMZ-Germany (catalogue no. ACC-660). The cell line was originally deposited at the University of Pittsburgh Medical Centre (DSMZ, 2015). SCC040 was established from a 50-year-old Caucasian male, non-smoker with a history of alcohol intake, who died of disease. These cells are primary, moderately differentiated tongue squamous cell carcinoma=; T2N2 and TNM stage 4 (Martin et al., 2008). The SCC040 cell line possesses wild-type p53. Doubling time was calculated to be between 21-25 hours (Dr Rachel Watkins, InHANSE).

VU147 (93-VU-147T) is a non-commercial HPV positive cell line, and was a kind gift from Dr Sally Roberts (University of Birmingham, West Midlands, UK). The cell line was originally deposited at the VU University Amsterdam (Hermsen et al., 1996). VU147 was established from a 58-year-old male, with a history of smoking and alcohol intake. These cells are primary, moderately differentiated floor of mouth SCC; T4N2 (Hermsen et al., 1996). VU147 cells contain HPV-16 DNA, and express HPV-16 E6/E7 viral oncoproteins (Rampias et al., 2009). Doubling time was calculated to be between 33-45 hours (Dr Rachel Watkins, InHANSE).

### *3.2.1.2 Cell culture protocol*

Cell culture medium was prepared using Dulbecco's Modified Eagle's Medium-HEPES Modification (DMEM- Sigma, D6171), and supplemented with 10% fetal bovine serum (FBS- Sigma, F7524), 1% L-Glutamine (2mM- Gibco, 25030-024), 1% Penicillin (50U/ml) with Streptomycin (50ug/ml)- (Gibco, 15070-063), and 1% (X100) non-essential amino acids (NEAA- Sigma, M7145).

Cells were maintained in T75 culture flasks (Corning Incorporated, 430641), at 37°C and 5% CO<sub>2</sub>. To prepare for counting or passaging cells were washed in PBS (phosphate-buffered saline), and re-suspended using TrypLE Express solution (1X, Gibco, 12605-010). Cell centrifuging was performed using a Sarstedt LC24 centrifuge (S/N 730513-7), and set at 1,100 rpm for 4 minutes to obtain a cell pellet. The cell pellet was re-suspended in DMEM and was split according to planned experiment requirements (e.g. 1:5). Cell passage was performed 2-3 times weekly, depending on cell confluence.

### *3.2.1.3 Counting cells*

In order to count cells 0.4% Trypan blue stain (Life Technologies, T10282) was used according to manufacturer's instructions. Cell counts were obtained using a Countess cell counting chamber slide (Invitrogen, C10283) and Countess automated cell counter (Invitrogen, C10281), which provided a digital reading of live cell count per ml. Viability of cells was typically 95% or above.

#### 3.2.1.4 Authentication of cells

The SCC040 cell line was authenticated in June 2014 by DNA profiling (DDC DNA Diagnostic Centre, London, UK). The VU147 cell line is not commercially available, and therefore could not be authenticated by DNA profiling. Instead PCR was performed to authenticate the HPV status of the two cell lines, and described below.

Cells were first prepared for counting (Section 3.2.1.3). Using the DNeasy Blood and Tissue Kit (Qiagen, 69504), steps were followed as per manufacturer's instructions. Briefly, 1ml of cell volume (maximum recommended  $5 \times 10^6$  cells) was transferred to a micro-tube and centrifuged in an Eppendorf Centrifuge 5424 (S/N 5424CJ050429) at 200 rpm for 5 minutes. Cell pellets were re-suspended in 200 $\mu$ l PBS, and supplemented with 20 $\mu$ l proteinase K and 200 $\mu$ l Buffer AL. The solution was mixed by vortexing using a Whirlmixer (Fisons Scientific Apparatus, WM/250/SC). Samples were then placed in a water bath at 56°C for 10 minutes. A further 200 $\mu$ l 100% ethanol was added and samples vortexed. The mixture was transferred to a DNeasy Mini spin column, placed within a 2ml collection tube. Tubes were centrifuged at 8000 rpm for 1 minute, and the spin column was transferred into a new collection tube. The spin column was treated similarly with Buffer AW1 then Buffer AW2. Finally, DNA was eluted by addition of Buffer AE and centrifuging.

DNA yield was quantified by sampling a 1 $\mu$ l volume using the Nanodrop NP-1000 Spectrophotometer (S/N 1705). The spectrophotometer was calibrated with the

eluting Buffer AE. Samples were analysed with the Nanodrop 3300 software (Thermo Fisher Scientific, Version 2.7.0).

The PCR reaction mixture included 12.5µl GoTaq Hot Start Green Master Mix (Promega, MF122), 30ng DNA template or water (negative control), 1.25µl forward primer (Table 3.1), 1.25µl reverse primer, and 9µl nuclease-free water (Promega, P119A). PCR controls included previously prepared and tested VU147 sample (HPV positive cell) as a positive control, and C33a sample (HPV negative cell) and water as negative controls. Since quantification of PCR product was not intended an endogenous positive control (e.g. GAPDH) was not included.

Forward primer	5'-AATGTTTCAGGACCCACAGG-3'
Reverse primer	5'-CCCGAAAAGCAAAGTCATATACC-3'

*Table 3.1: HPV 16 E6 primers (Sigma), kindly provided by Dr Steven Lee (University of Birmingham, UK)*

The PCR reaction was performed using the Geneamp PCR System 9700 (PE Applied Biosystems). Conditions were set as follows: 95°C for 15 minutes; 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 40 seconds; 72°C for 10 minutes; 4°C hold. Gel electrophoresis was prepared in 2% UltraPore Agarose (Invitrogen, 16500\_500) added to 50ml of 1X TAE (Tris-acetate-EDTA) buffer, with the addition of 5µl SYBR Safe DNA gel stain (Invitrogen, 533102). Each 10µl PCR sample was supplemented with 2µl Gel Loading Dye Blue (Biolabs, B7021S). In addition a 100bp DNA ladder (Biolabs, N3231S) was run with the samples, and gel electrophoresis performed at 110 volts for 30 minutes. An image of the gel was taken



under UV light using the Geneflow Geneflask (S/N 1055) and Pulnix TM-300 camera (S/N 002342).

### 3.2.2 Drug Targets

#### 3.2.2.1 Initial screening

Drug targets were screened from a drug library (FMC) kindly provided by Dr Farhat Khanim (University of Birmingham, West Midlands, UK), which included 100 off-patent licenced drugs. Initial screening was performed by robotic HTS using proliferation assays (Dr Peter Rae, InHANSE), to identify target candidates. The results were standardised according to effect on proliferation compared to untreated control, and graded according to efficacy (Table 3.2). Drug targets were selected if they had moderate efficacy or better, and were effective in the selected cell lines.

<b>Efficacy</b>	<b>% Reduction in proliferation vs. untreated control</b>
Increased proliferation effect	<0%
Poor/No effect	0-25%
Moderate effect	26-50%
Good effect	51-75%
Considerable effect	76-100%

*Table 3.2: Effect of drug target on proliferation compared to untreated control*

#### 3.2.2.2 Preparing drugs

Drug targets to be assessed were pre-prepared by Dr Peter Rae (InHANSE) into X10,000 stock aliquots, and stored at -20°C until required. During experiments all target drugs underwent serial dilutions using DMEM to make X1 final solution, which corresponded to peak serum concentration (clinically achievable concentration).

INH001 (Sigma) was prepared to a 950mM stock in water, and diluted to a 95 $\mu$ M working solution. INH002 (Sigma) was prepared as a 57.2mM stock in DMSO (Dimethyl-Sulfoxide Hybri-Max, Sigma- D2650), and diluted to 5.72 $\mu$ M working solution. INH003 (Sigma) was prepared as a 32mM stock in ethanol, and had a working concentration of 3.2 $\mu$ M.

Cisplatin was included as a positive control and allows comparison to standard treatment. During experiments 3mg Cisplatin powder (Biovision, 1550-1000) was dissolved in 1ml of DMSO to form a 10,000 $\mu$ M solution. Serial dilutions were performed in DMEM to a final concentration of 5 $\mu$ M, 10 $\mu$ M or 15 $\mu$ M solutions. These Cisplatin concentrations were selected based on IC<sub>50</sub> data and previous optimisation experiments performed on SCC040 and VU147 cells for the Accelerated project (InHANSE).

### **3.2.3 Proliferation Assay**

#### *3.2.3.1 Optimisation*

The alamarBlue (Invitrogen, DAL1100-463418) cell viability assay was utilised to quantitatively measure proliferation of the cells. This assay measures the conversion of Resazurin (blue, weakly fluorescent) to Resorufin (red, highly fluorescent), which is proportional to cell activity (alamarBlue, 2015). The assay was initially optimised, to determine the appropriate conditions for each cell line (Accelerated, InHANSE). Briefly, this involved plating cells at various seeding

densities (range 800 to 5000 cells/well) in 96 well plates, to determine which density will grow to 80-90% confluence by 72 hours. The 72-hour time-point was selected as an appropriate end-point, in order to adequately assess drug treatment effects in subsequent experiments. Cells were pre-seeded the day before. Three independent laboratory staff performed optimisation, and all densities performed in triplicate. Optimum cell density for SCC040 was found to be 2,500 cell/well, and VU147 was 4000 cells/well.

Furthermore, alamarBlue reagent was incubated at various time-points (2, 4, 6, 8 hours), to determine appropriate RFU (relative fluorescence units) readings. The 4-hour incubation period was subsequently chosen for both cell lines.

### *3.2.3.2 Protocol*

Cells were first prepared for counting (Section 3.2.1.3). Live cell counts were used to calculate serial dilutions to obtain the final cell densities. Cells were pre-seeded in a Costar 96 well plate (Corning Incorporated, 3596) the day before. A 100µl volume was pipetted into corresponding wells into 3 separate plates (24, 48, 72 hours plates). A further 3 plates were seeded with DMEM only, in order to measure alamarBlue background readings to calculate the effect of drugs only on plate readings.

On day two fresh drug and vehicle-only controls dilutions were performed to obtain a X2 (two times the final concentration) solution. Cisplatin was freshly prepared (Section 3.2.2.2), and diluted to provide X2 working solution (10µM, 20µM,

30 $\mu$ M). For each plate, 100 $\mu$ l of DMEM was added to the first column, as a negative control. Corresponding columns were then treated with each condition, with the addition of 100 $\mu$ l volume, with a final drug/control concentration of X1 (final well volume = 200 $\mu$ l). Each condition had triplicate wells. Plates were then incubated for 24, 48 or 72 hours.

Following incubation, 20 $\mu$ l of alamarBlue reagent was added to the required plates 4 hours prior to the end-point (20 hours > 24 hours, 44 hours > 48 hours, 68 hours > 72 hours) as per the manufacturers instructions. The lids were then removed from the plates and placed in the Wallac 1420 Victor<sup>2</sup> Multilabel Counter (S/N 29981517). Plates were read at 550/590nm 0.1s fluorescence using the Wallac 1420 Manager (Perkin Elmer Life Sciences, Version 3.00.0.53). This provided relative fluorescence values (RFU). All values were adjusted by deducting the results from the media-only plates, which measured background alamarBlue values. Using the triplicate results for each condition an average value was calculated for the technical repeats. The average results were then converted to a percentage against the untreated control.

Experiments were repeated on three separate occasions to provide biological/experimental repeats, and an average calculated from percentage values.

### **3.2.4 Migration Assay**

#### *3.2.4.1 Optimisation*

The migration assay, also known as wound healing or scratch wound assay, was optimised to determine the appropriate seeding cell density for each cell line. In order to perform the assay ~100% confluence was required at 48-hour incubation, to assess migration once a wound was created.

Cells were prepared for counting (Section 3.2.1.3), and serial dilutions performed to obtain final cell densities ranging from 50,000 cells/well to 300,000 cells/well (50,000 increments). Seeding of cells was performed in a Costar 12 well plate (Corning Incorporated, 3513). Triplicate wells were set up for each cell density. Plates were incubated and representative images taken at 24, 48, 72 and 96 hours. To achieve 100% confluence at 48 hours, the required seeding density for SCC040 was 250,000 cells/ml. VU147 cells did not appear to reach 100% confluence by 48 hours during optimisation, which could partly be due to the increased doubling time for this cell line. Therefore the migration assay could not be performed on VU147 cells.

### *3.2.4.2 Protocol*

Cells were prepared for counting (Section 3.2.1.3). Serial dilutions were performed to obtain a final SCC040 cell concentration of 250,000 cells/ml. Seeding of cells with 1ml cell volume was performed in a 12-well plate. After 24 hours cell confluence was assessed to ensure ~100% confluence. The media was then replaced with fresh media, and X2 drug dilutions and controls were prepared. To each corresponding well 1ml of drug or control was added to a final concentration of X1. Plates were incubated for 24 hours.

Following incubation wounds were created using a sterile 10µl pipette tip, and changed between wells. Media and drugs/controls were then replaced to minimise cell debris within the wells. Imaging of plates was carried out using a Nikon Eclipse TS100 microscope (10x magnification), Q-Imaging Rolera-XR camera and Q-Capture software (2011 Quantitative Imaging Corporation, Version 2.9.13). Prior to imaging 3 points were marked on the underside of each well using a permanent marker pen, to ensure accurate and reproducible points to image. Representative images were taken at three time points (0, 6, 24 hours). The final image was performed once the media-only control wells appeared to show complete wound closure, at approximately 24 hours.

Images of wounds were analysed using Image J (National Institute of Health, USA, Version 1.48), which enabled measurement of wound areas. First an average value for each well was calculated, and then an average from the technical repeats. Experiments were repeated on three separate occasions to provide biological repeats, and an average calculated. The final averages were used to analyse wound closure at 24 hours against 0 hours, to provide percentage wound closure at 24 hours for each biological repeat. Percentages were averaged (N=3) and adjusted against untreated control.

### **3.2.5 Cell cycle Flow Cytometry Assay**

#### *3.2.5.1 Optimisation*

Flow cytometry was utilised to perform Propidium Iodide (PI) cell cycle analysis. Optimisation for each cell line was required to determine the appropriate seeding density. The ideal density for cell cycle analysis would enable adequate cell growth but avoid over-confluence (approximately 70% confluence).

Cells were prepared for counting (Section 3.2.1.3), and serial dilutions performed to obtain final cell densities ranging from 50,000 cells/well to 300,000 cells/well (50,000 increments). Seeding of cells was performed in a Costar 6 well plate (Corning Incorporated, 3516). Triplicate wells were set up for each cell density. Plates were incubated and representative images taken at 24, 48, 72 and 96 hours. Selected seeding density for SCC040 was 100,000 cells/well (50,000 cells/ml), and was optimised by Dr Rachel Watkins (InHANSE). The cell density for VU147 was 150,000 cells/well (75,000 cells/ml).

Optimisation of time-points for drug treatment was performed (16, 24, 48, 72 hours). These time-points were chosen based on the optimisation and results of the proliferation assay. The final selected time points were 16 and 72 hours. The 16-hour time-point showed no significant effect on the cell cycle analysis with both the drugs and positive controls, whereas 72 hours allowed sufficient time for target drugs to demonstrate an effect.

### *3.2.5.2 Protocol*

Cells were prepared for counting (Section 3.2.1.3). Serial dilutions were performed to obtain a final cell concentration as described above. Seeding of cells

with 2ml cell volume was performed in a 6-well plate, and incubated for 24 hours. Media was then replaced with 1ml fresh media, and supplemented with 1ml of X2 drug dilution or control, with 3 wells per condition. Cisplatin 15 $\mu$ M was selected as a positive control. Plates were incubated for either 16 or 72 hours, according to experiment.

Cell cycle buffer was freshly prepared in a 15ml centrifuge tube. This consisted of 5ml sterile distilled water, 5 $\mu$ l 0.1M Sodium Chloride (final concentration 0.1mM), 50 $\mu$ l Triton X-100 (Sigma, T8787), and 50 $\mu$ l of 1mg/ml Propidium Iodide solution (Sigma, P4864). The Propidium Iodide allowed measure of DNA content within samples during analysis.

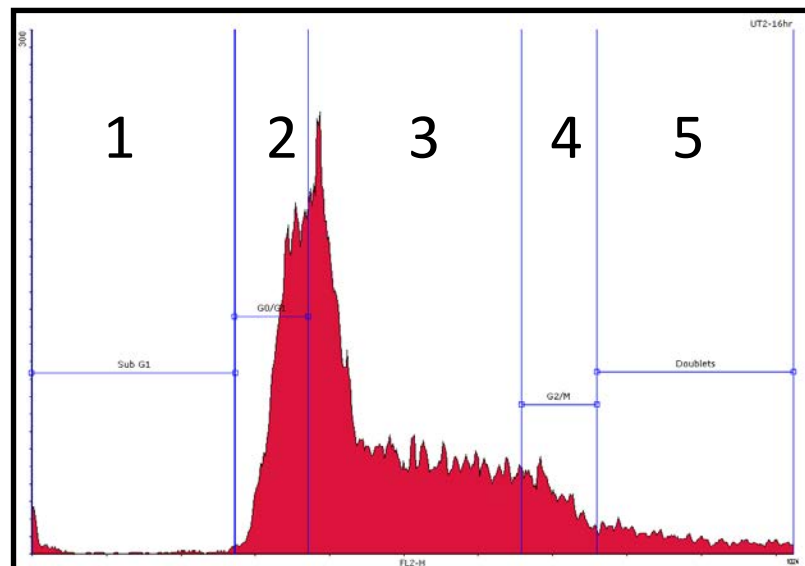
Following the required incubation period cells were then harvested. The media, from the 3 wells of each condition, were collated into a 15ml centrifuge tube and labelled. Cells were washed with 1ml PBS and 500 $\mu$ l TrypLE added to each well. Plates were incubated for 5-10 minutes until all cells had detached. The volume from 3 wells was added to the corresponding 15ml tube. The tubes were then centrifuged at 1,100 rpm for 4 minutes to obtain cell pellets, and the supernatant carefully discarded. Cell pellets were re-suspended using 500 $\mu$ l cell cycle buffer and the volume transferred to 5ml Culture Tubes (VWR, 60818-486). Samples were stored for 1-hour at 4°C and protected from light with aluminium foil.

Flow cytometry analysis was performed using the BD FACSCaliber (S/N E571) and BD Cell Quest software, which was kindly provided by Dr Farhat Khanim (University of Birmingham, West Midlands, UK). Samples were vortexed prior to



analysis, and were run to record 20,000 events per sample, and set to measure fluorescence using the FL2-H channel. This provided a histogram demonstrating phases of the cell cycle.

Further analysis was performed using Flowing Software (Perttu Terho, University of Turku, version 2.5.1). This measured highlighted areas of a histogram, representing stages of the cell cycle as percentages (Figure 3.2). However, since the S-phase graphically overlaps into the G0/G1 and G2/M peaks these two phases are calculated by measuring one half of their peaks and multiplying by two. The S-phase was indirectly measured by subtracting measured values from 100 [S-phase (%) =  $100 - (\% \text{ Sub G1}) - (\% >2N) - 2(\% \frac{1}{2} \text{ G0/G1}) - 2(\% \frac{1}{2} \text{ G2/M})$ ]. Experiments were repeated on three separate occasions to provide biological repeats, and averages calculated as percentages of cell cycle stages.



*Figure 3.2: Example flow cytometry histogram labelled with phases of cell cycle. 1- Sub G1 area, 2-  $\frac{1}{2}$  G0/G1 area, 3- S-phase area (overlapping G0/G1 and G2/M areas), 4-  $\frac{1}{2}$  G2/M area, 5- >2N area*

### 3.2.6 Clonogenic Assay

#### 3.2.6.1 Optimisation

The clonogenic assay was utilised to assess the effect on colony formation with target drugs and controls. In principal, cells are seeded at densities low enough to allow single cells to emerge and incubated until adequate colonies have formed. Colonies are defined as individual cell clusters containing 50 or more cells (Rafehi et al., 2011, Franken et al., 2006). Initial optimisation and protocol was performed (Dr Rachel Watkins, InHANSE), and was adapted from a protocol by Franken et al (Franken et al., 2006). The ideal incubation period was determined to be between 10-14 days.

Optimisation for seeding density was performed for SCC040, and required adjustments for further repeats, with three in total. Each drug or control required optimisation, and this required predicting the cell's response to each treatment by analysing the effects seen in the other experiments and increasing or decreasing seeding density accordingly. Differences in seeding densities were taken into account in the data analysis.

An initial clonogenic optimisation assay was performed for VU147, and included only untreated control, DMSO, Cisplatin 1 $\mu$ M and IR 1gy. However, the VU147 cells did not appear to form adequate colonies, and therefore no further repeats were attempted. Some cancer cell lines, including HNC cells, possess little or no clonogenicity, such as UM-SCC-29 (Tang et al., 2013).

### 3.2.6.2 Protocol

Cells were prepared for counting (Section 3.2.1.3). Serial dilutions were performed to obtain an initial stock cell concentration of 2,500-cells/ml. Using this stock, further dilutions were prepared to required volumes and cell densities. A 5ml volume was removed from tubes containing densities for planned irradiation. Irradiation therapy was performed at 1 Gy. In 6 well plates a 2ml cell volume of the three seeding densities was prepared, with 2 wells per density, and incubated for 24 hours.

Drug and control dilutions were then prepared to X2 concentration. Each plate was supplemented with 2ml/well of the required treatment to a final X1 concentration, and 2ml/well media added to the IR plate. Plates were incubated for a further 24 hours. The media and drug/control were removed and then replaced with 4ml media per well. Plates were incubated and monitored until colony formation (>50 cells/colony) was visible in the untreated control (10-14 days).

Staining of cells was performed using Crystal violet staining solution. Media was discarded, and wells washed with PBS solution. Crystal violet was added to cover the surface of each well. The staining solution was removed, and each plate was washed with tap water.

Plates were scanned using the HP Scanjet 5590P. Images were analysed with Image J, and colonies counted by a pre-programmed Colony Counter macro kindly

provided by Dr Peter Rae (InHANSE). Only one cell density was analysed for each condition, and was chosen according to their clonogenic appearance. Ideally the selected density resulted in neither under nor over confluent wells, with clearly demarcated colonies. Average values were calculated for the selected cell density. Calculations were then performed to determine plating efficiency for each condition, and surviving fractions as follows (Franken et al., 2006):

$$\text{Plating Efficiency \% (PE)} = (\text{Number of colonies formed} / \text{Number of cells seeded}) \times 100$$

$$\text{Surviving Fraction \% (SF)} = (\text{PE of treatment} / \text{PE control}) \times 100$$

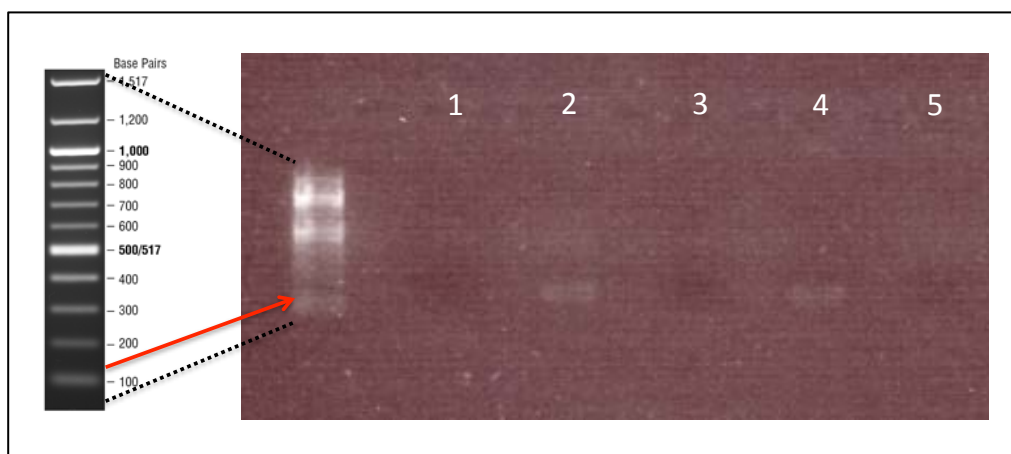
### 3.2.7 Statistical Analysis

Data were first analysed using Microsoft Excel for Mac 2011 (Version 14.0.0). Statistical analysis was performed with the GraphPad software (Prism, Version 6). The proliferation assay data was assessed by two-way ANOVA and post-hoc Tukey's multiple comparisons test. The migration and clonogenic assays were analysed by one-way ANOVA and post-hoc Dunnett's multiple comparisons test. The flow cytometry data was analysed by one-way ANOVA and post-hoc Tukey's multiple comparisons test. The results of the post-hoc tests were reported, with adjusted p values. Statistical significance was considered at  $p < 0.05$ .

### 3.3 Results

#### 3.3.1 Authentication of HPV status

In order to authenticate the HPV status of the SCC040 and VU147 cell lines PCR testing was utilised. The HPV 16 E6 primer was expected to yield a PCR product of approximately 146bp. The result in Figure 3.3 confirms the HPV positive status of the VU147 cell line, yielding a PCR product between 100 to 200bp.



*Figure 3.3: PCR results of HPV 16 status. Lane 1=SCC040, Lane 2=VU147, Lane 3=Water only control, Lane 4=Positive control (previously tested VU147 sample), Lane 5=Negative control (C33a sample). Fluorescence signal visible in lanes 2 and 4; corresponding between 100 to 200bp on the DNA ladder*

#### 3.3.2 Proliferation assay

##### 3.3.2.1 SCC040 cell line

The initial HTS had identified several potential drug targets, and 3 candidates were selected and validated using the alamarBlue proliferation assay. The effect on proliferation with each drug and controls were compared to the untreated control

(Figure 3.4). The statistically significant results are illustrated in Figure 3.5. INH001 significantly reduced proliferation at 24 hours (64.4%  $\pm$  3.5,  $p < 0.01$ ,  $N=3$ ), 48 hours (69.9%  $\pm$  11.5,  $p < 0.001$ ,  $N=3$ ), and 72 hours (75.4%  $\pm$  8.9,  $p < 0.001$ ,  $N=3$ ). INH003 also demonstrated significant reduction at 24 hours (66.5%  $\pm$  1.7,  $p < 0.01$ ,  $N=3$ ), 48 hours (81.1%  $\pm$  5.9,  $p < 0.0001$ ,  $N=3$ ), and 72 hours (93.2%  $\pm$  0.4,  $p < 0.0001$ ,  $N=3$ ). However, INH002 and vehicle controls showed no significant effect on proliferation at these time points. The results of INH001 and INH002 were similar to the Cisplatin controls. Interestingly the Cisplatin controls did not significantly effect proliferation at 24 hours, but were more effective at 48 hours and 72 hours. For example, Cisplatin 5 $\mu$ M reduced proliferation by 40.3% ( $\pm$  12.7,  $N=3$ ) at 24 hours, 72.7% ( $\pm$  6.9,  $p < 0.001$ ,  $N=3$ ) at 48 hours, and 82.7% ( $\pm$  2.2,  $p < 0.0001$ ,  $N=3$ ) at 72 hours.

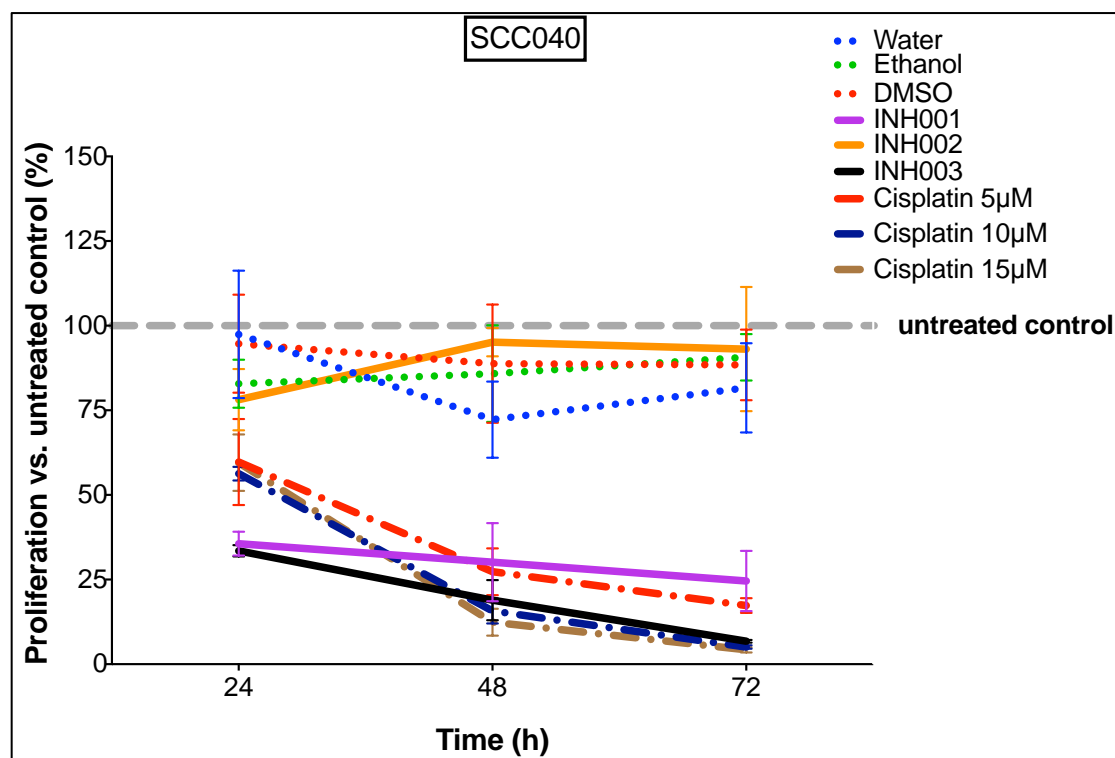


Figure 3.4: Percentage proliferation of SCC040 cells with target drugs (solid lines), positive controls (dashed) and vehicle controls (dotted) at three time points, including standard error of means. Treatments were compared against untreated control (grey dashed line)

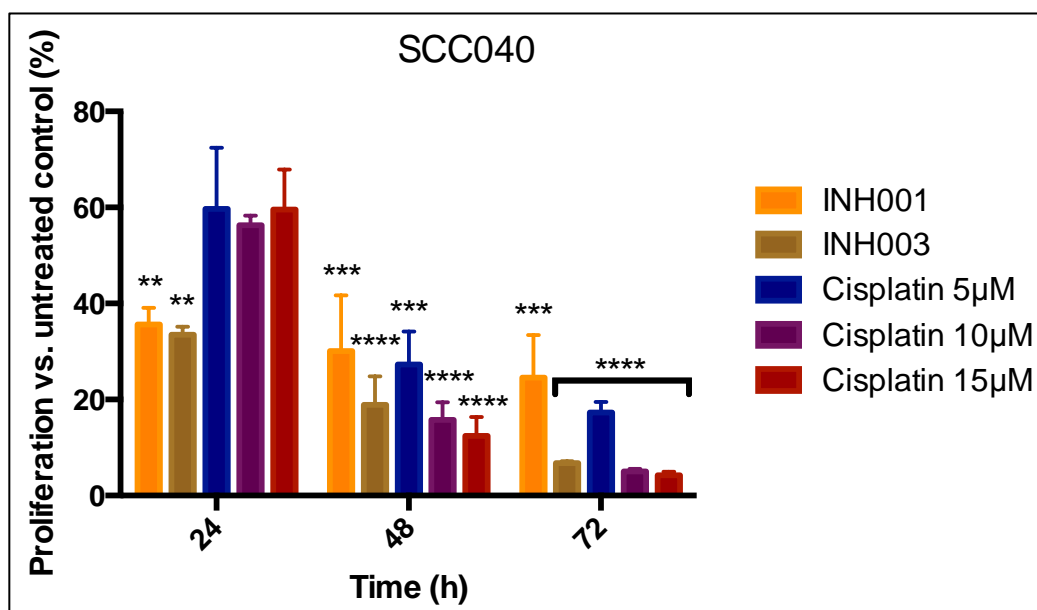


Figure 3.5: Treatments demonstrating statistically significant effects on SCC040 cells with percentage proliferation compared to untreated control, including standard error of means. Statistical analysis performed with two-way (time and treatment) ANOVA and Tukey's post-hoc multiple comparisons test. (N=3; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ )

### 3.3.2.2 VU147 cell line

The proliferation assay results for VU147 are demonstrated in Figure 3.6, and statistically significant outcomes in Figure 3.7. At 24 hours none of the treatments or controls had a significant effect on proliferation. INH003 showed statistically significant reduction in proliferation at 48 hours (65.2%  $\pm$  8.1,  $p < 0.05$ , N=3), and 72 hours (85.9%  $\pm$  1.7,  $p < 0.001$ , N=3). This was similar to Cisplatin 15µM at 48 hours (69.4%  $\pm$  3.4,  $p < 0.05$ , N=3), and 72 hours (84.5%  $\pm$  4.2,  $p < 0.001$ , N=3). Neither INH001 nor INH002 demonstrated a significant effect on proliferation.

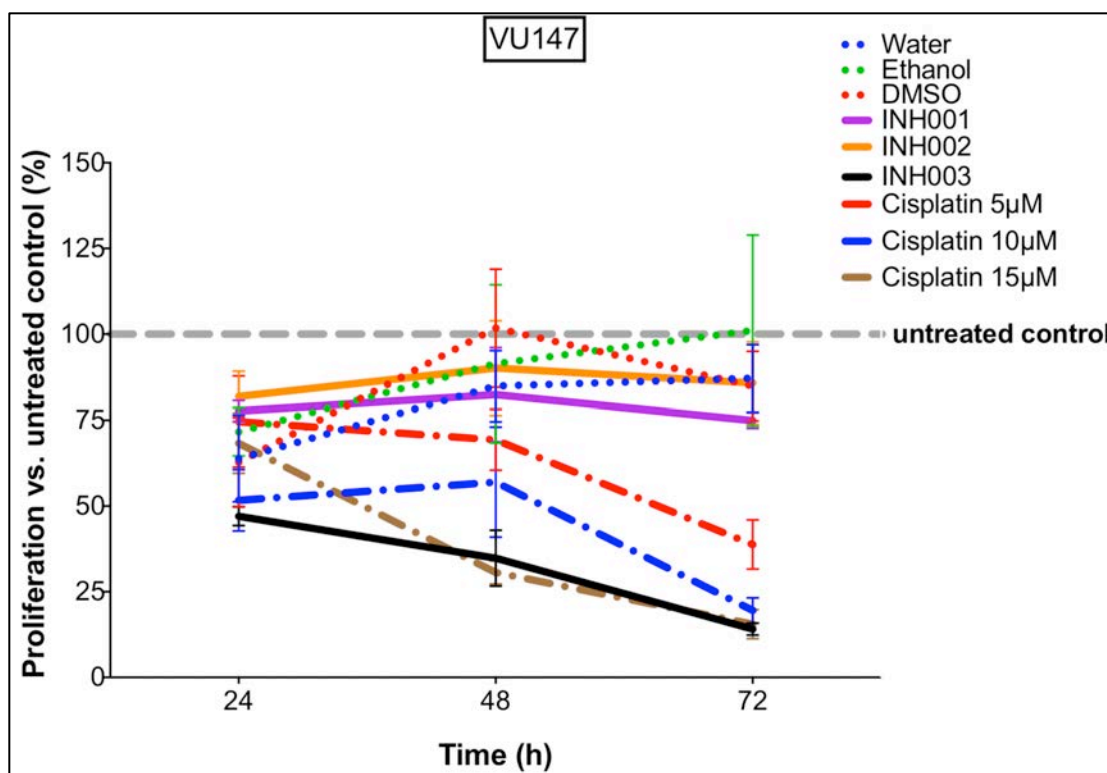


Figure 3.6: Percentage proliferation of VU147 cells with target drugs (solid lines), positive controls (dashed) and vehicle controls (dotted) at three time points, including standard error of means. Treatments were compared against untreated control (grey dashed line)

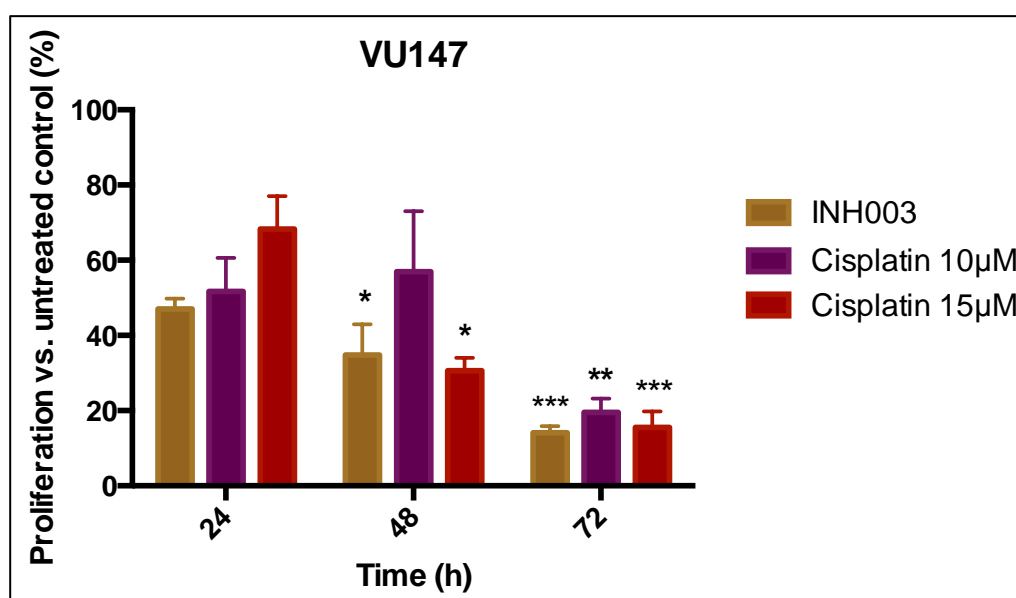


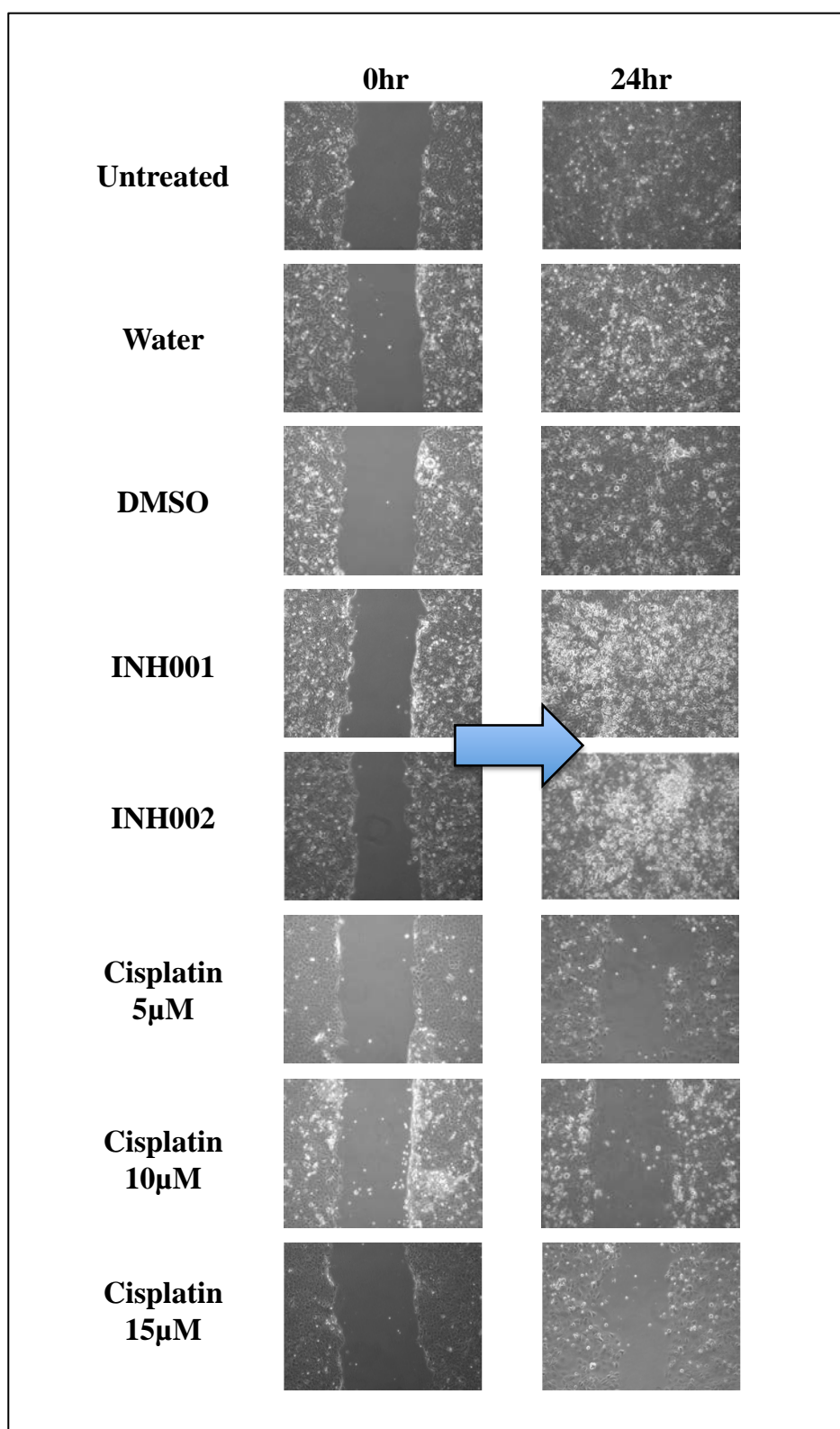
Figure 3.7: Treatments demonstrating statistically significant effects on VU147 cells with percentage proliferation compared to untreated control, including standard error of means. Statistical analysis performed with two-way (time and treatment) ANOVA and Tukey's post-hoc multiple comparisons test. ( $N=3$ ; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ )



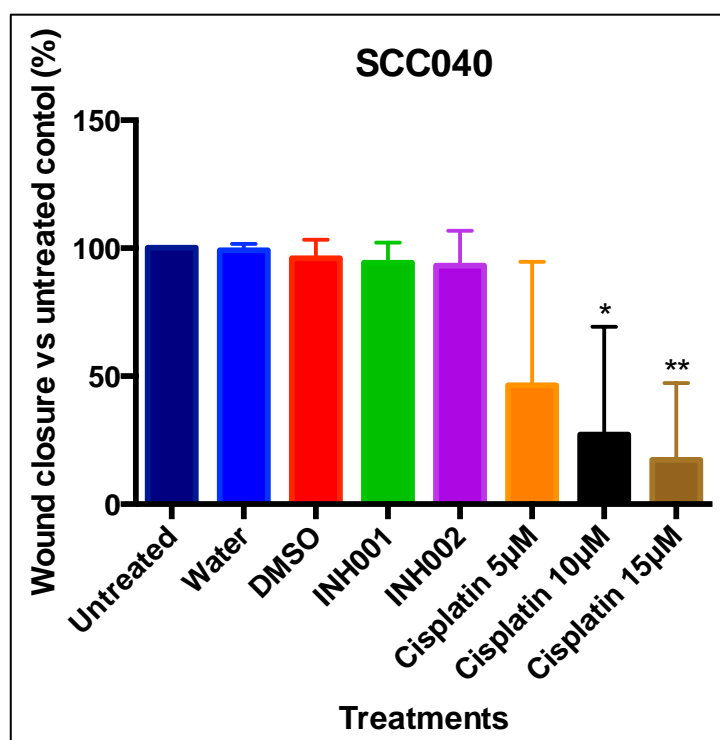
### 3.3.3 Migration assay

#### 3.3.3.1 SCC040 cell line

The migration assay was performed using the scratch wound method and included INH001 and INH002. This assay was utilised at an early stage in our experiments, prior to the inclusion of INH003 and Ethanol control. Representative images (Figure 3.8) were taken after creating the wounds and again at 24 hours (time point of wound closure for untreated control). Both INH001 and INH002 appeared to show no significant effect on wound closure, and were comparable to untreated and vehicle-only controls. Cisplatin controls did demonstrate significantly reduced wound closure rates compared to untreated control (Figure 3.9). Cisplatin 5 $\mu$ M reduced wound closure by 53.6% (+/- 27.9, N=3), Cisplatin 10 $\mu$ M by 72.8% (+/- 24.3,  $p < 0.05$ , N=3), and Cisplatin 15 $\mu$ M by 82.3% (+/- 17.3,  $p < 0.01$ , N=3).



*Figure 3.8: Migration assay for SCC040 cells treated with drug targets, vehicle-only controls and Cisplatin controls. Representative images were taken at 0 hours and 24 hours, and demonstrate complete wound closure for all conditions apart from Cisplatin controls*



*Figure 3.9: Percentage wound closure in SCC040 cells treated with drug targets and controls compared to untreated control, including standard error of means. Statistical analysis performed with one-way ANOVA and Dunnett's post-hoc multiple comparisons test.*

*(N=3; \* $p < 0.05$ , \*\* $p < 0.01$ )*

### 3.3.3.2 VU147 cell line

The optimisation experiment for VU147 did not achieve 100% confluence by 48 hours using a range of seeding densities (50,000 to 300,000 cells/well). In comparison the SCC040 cells reached 100% confluence by 48 hours when seeded at 250,000 cells/well (Figure 3.10). This was essential prior to creating a wound, in order to assess migration of cells into the wound only. Therefore the migration assay was not performed for VU147 cells.

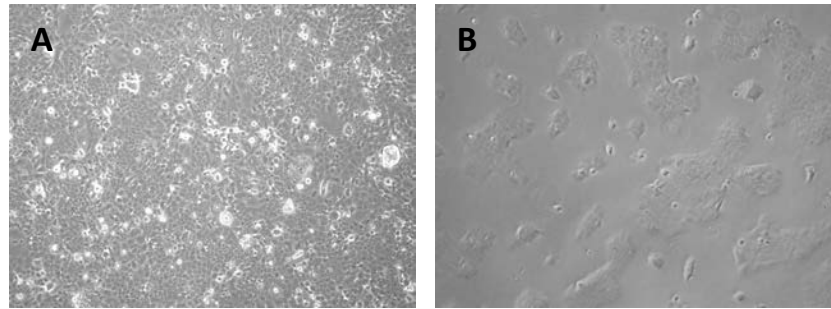


Figure 3.10: Representative images of both cell lines seeded at 250,000 cells/well at 48 hours incubation in a 12-well plate: A) SCC040 cells at 100% confluence, B) VU147 cells not 100% confluent

### 3.3.4 Flow cytometry

#### 3.3.4.1 SCC040 16 hours

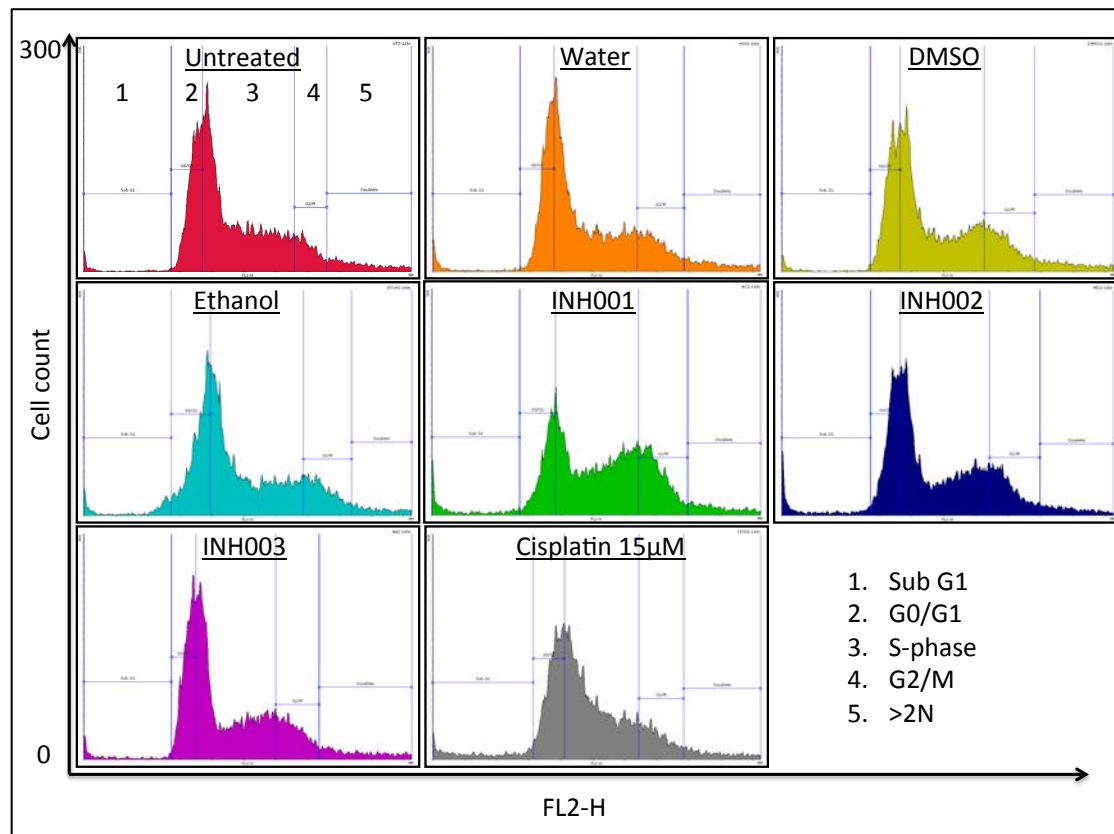
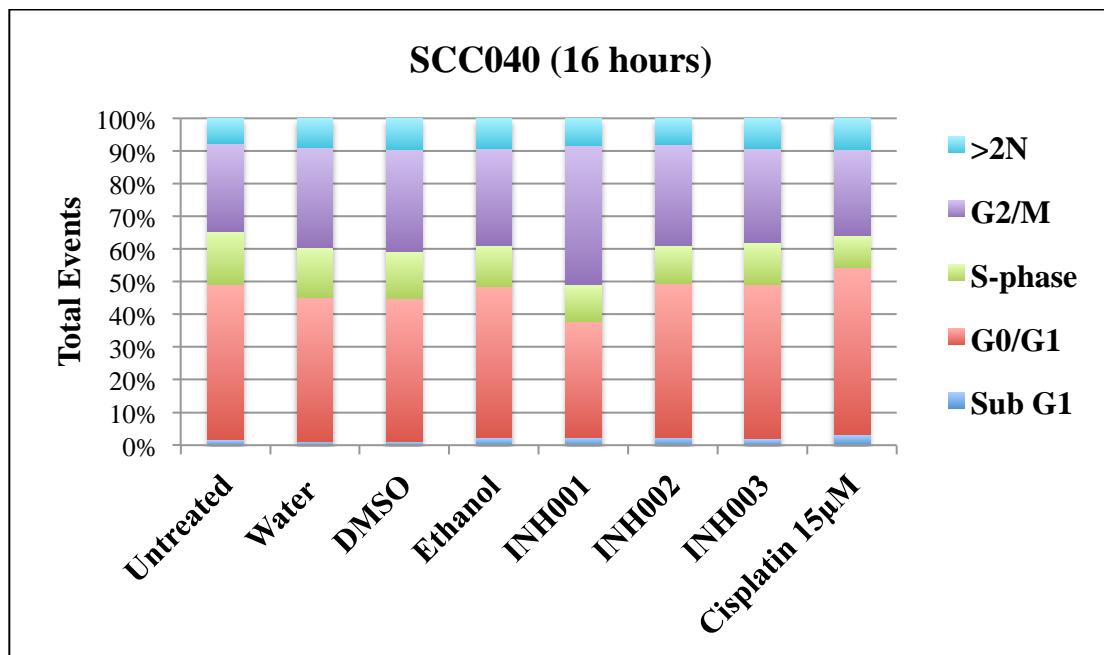


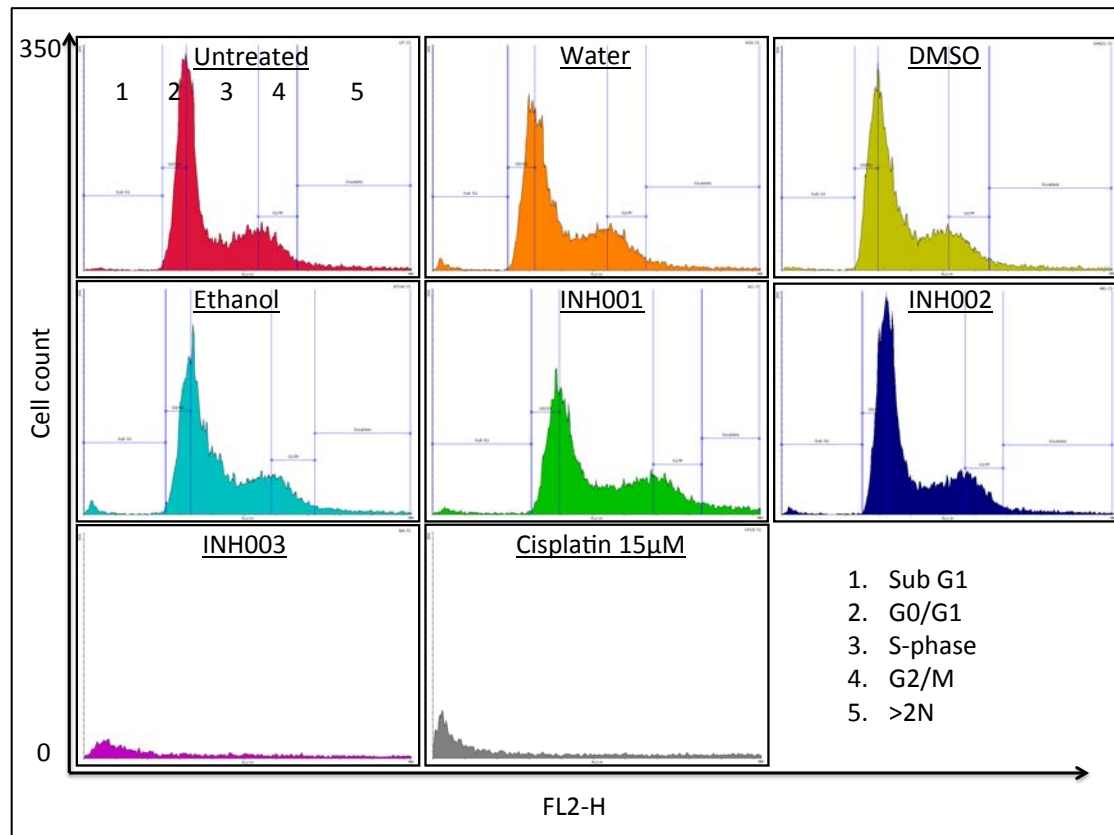
Figure 3.11: Histograms representing flow cytometry analysis at 16 hours for SCC040 cells with treatments and controls. The y-axis represents cell count and the x-axis represents Propidium Iodide relative fluorescence intensity measured via the FL2-H channel. The phases of the cell cycle are gated in order to measure area under the graph as a percentage of overall events (numbered 1-5)

Flow cytometry was performed using Propidium Iodide to measure DNA content in cells, which is proportional to fluorescent intensity. The data was converted into histograms (Figure 3.11) to represent stages of the cell cycle. At 16 hours treatment the SCC040 cells demonstrated no significant change in histogram shape between all the conditions. The histograms were gated to calculate the percentage total events for each stage of the cell cycle (Figure 3.12). There was no statistically significant difference between the drug targets and controls after analysing with one-way ANOVA and post-hoc Tukey's multiple comparisons test.



*Figure 3.12: Percentage total events for each stage of the cell cycle at 16 hours treatment for SCC040 cells*

## 3.3.4.2 SCC040 72 hours



*Figure 3.13: Histograms representing flow cytometry analysis at 72 hours for SCC040 cells with treatments and controls. Both INH003 and Cisplatin control demonstrated a cytotoxic effect on the cells*

At 72 hours treatment of SCC040 cells both INH003 and Cisplatin control killed the cells, as demonstrated by the loss of normal cell cycle appearance in the histograms and peak in the Sub G1 area (Figure 3.13). However, INH001 and INH002 appeared to show a normal cell cycle graph with no significant effect on the percentage total events in the stages of the cell cycle (Figure 3.14).

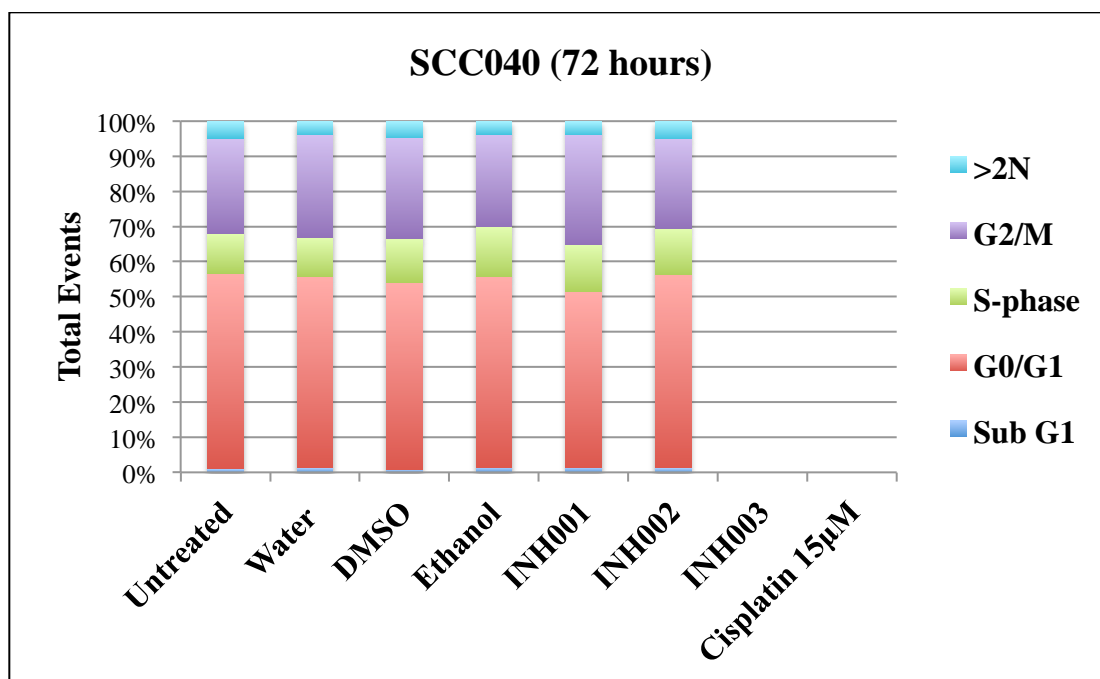


Figure 3.14: Percentage total events for each stage of the cell cycle at 72 hours treatment for SCC040 cells

#### 3.3.4.3 VU147 16 hours

At the 16 hours treatment the VU147 cells did not demonstrate any significant change in the cell cycle histograms with the drug targets or controls (Figure 3.15). Comparing the percentage of total events at each cell cycle stage, there was no statistically significant difference between the various conditions (Figure 3.16).

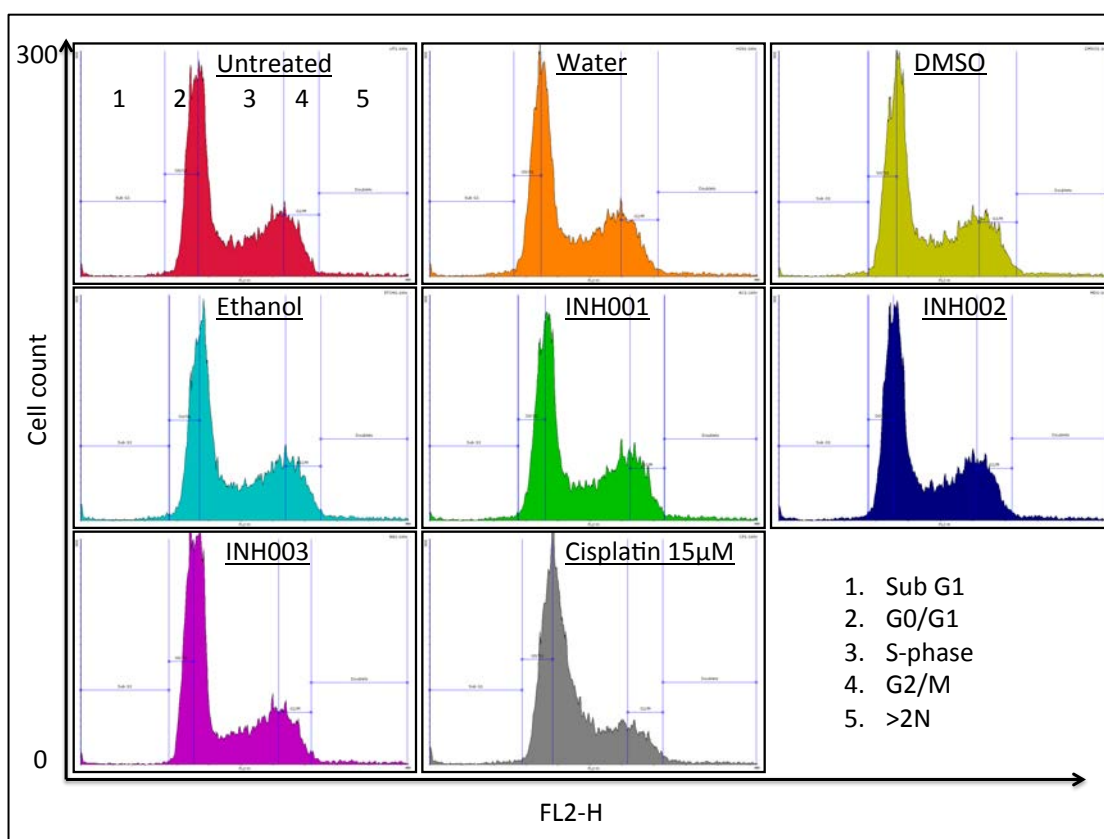


Figure 3.15: Histograms representing flow cytometry analysis at 16 hours for VU147 cells with treatments and controls

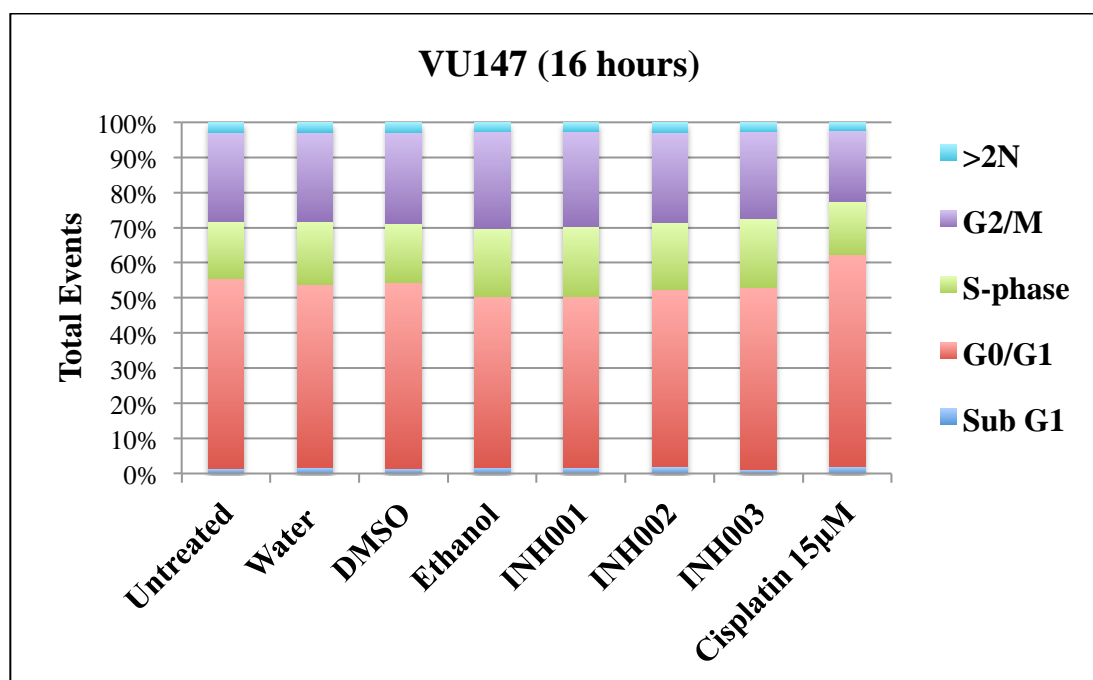


Figure 3.16: Percentage total events for each stage of the cell cycle at 16 hours treatment for VU147 cells



## 3.3.4.4 VU147 72 hours

The cell cycle analysis for VU147 at 72 hours demonstrated no significant change in histograms between the drug targets and vehicle controls (Figure 3.17). Only Cisplatin 15 $\mu$ M control altered the appearance of the cell cycle histogram, with an increase percentage of Sub G1 and G2/M areas and reduction in G0/G1 peak (Figure 3.18). Comparing to untreated control Cisplatin showed a statistically significant difference in Sub G1 ( $p < 0.0001$ ), G0/G1 ( $p < 0.0001$ ), and G2/M ( $p < 0.05$ ). The Sub G1 area represents fragmented DNA and debris, and therefore apoptotic cells. The increase in G2/M peak and reduction in G0/G1 suggests arrest of cell cycle at the G2/M phase.

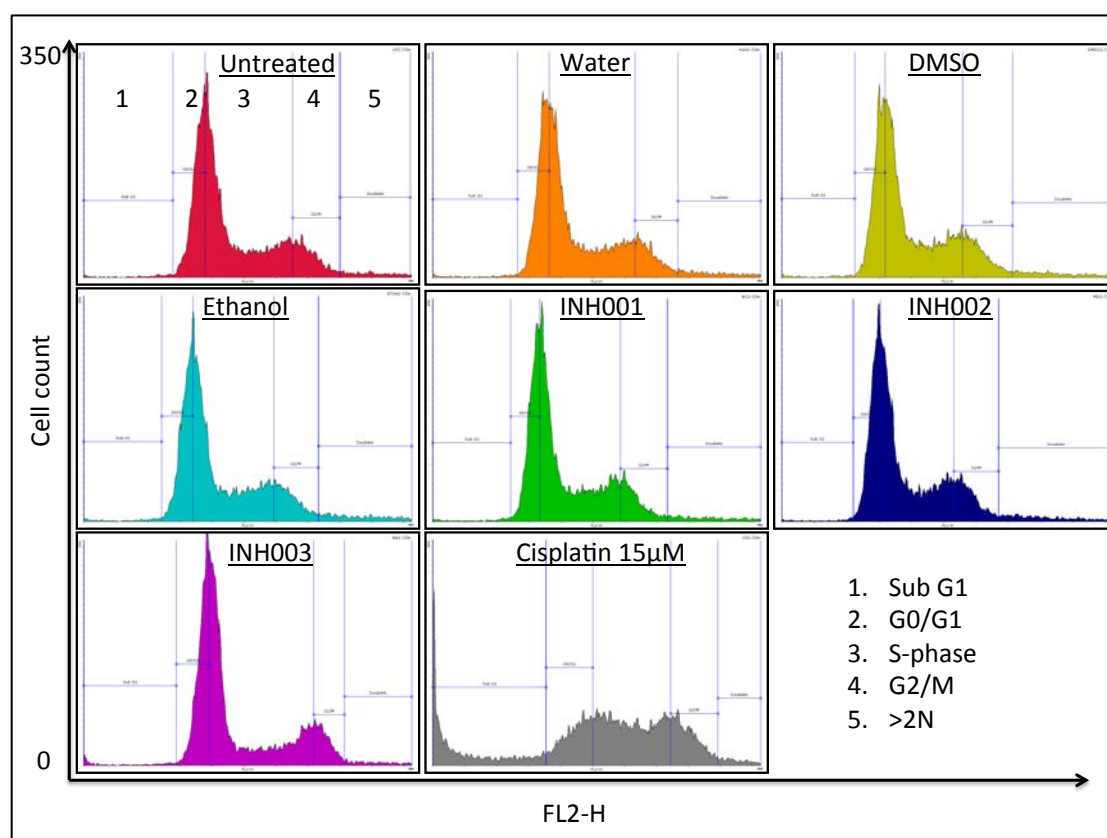


Figure 3.17: Histograms representing flow cytometry analysis at 72 hours for VU147 cells with treatments and controls. Only Cisplatin control appeared to alter the appearance of the cell cycle, with an increase in Sub G1 and G2/M areas, and a reduction in the G0/G1 peak

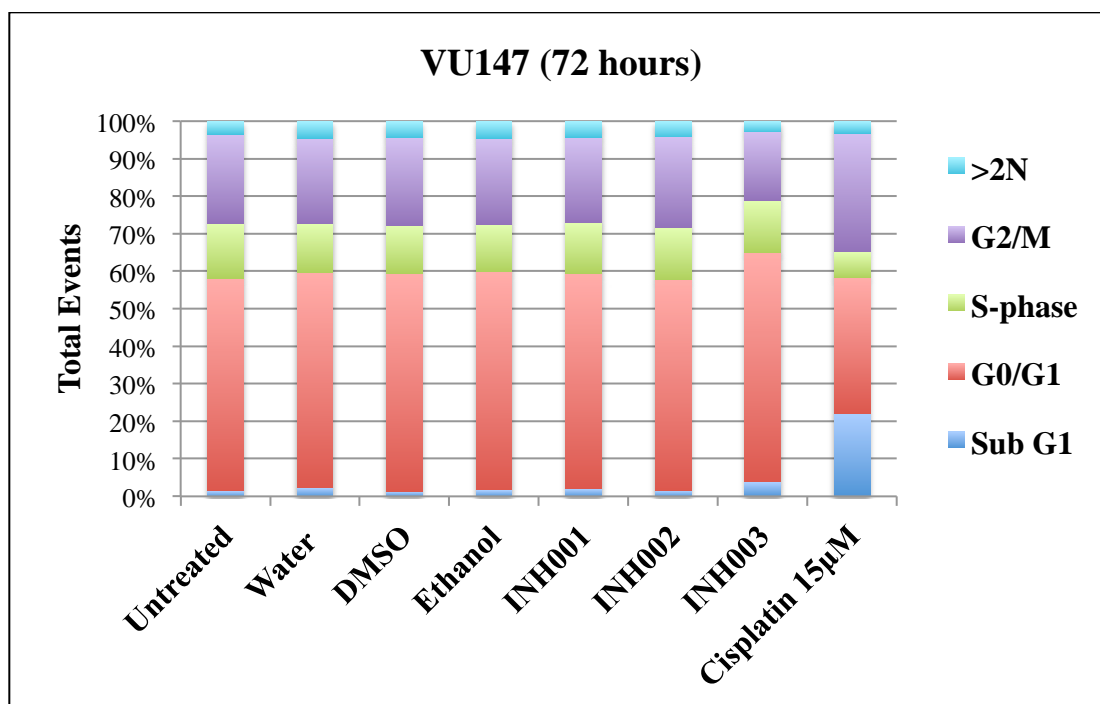


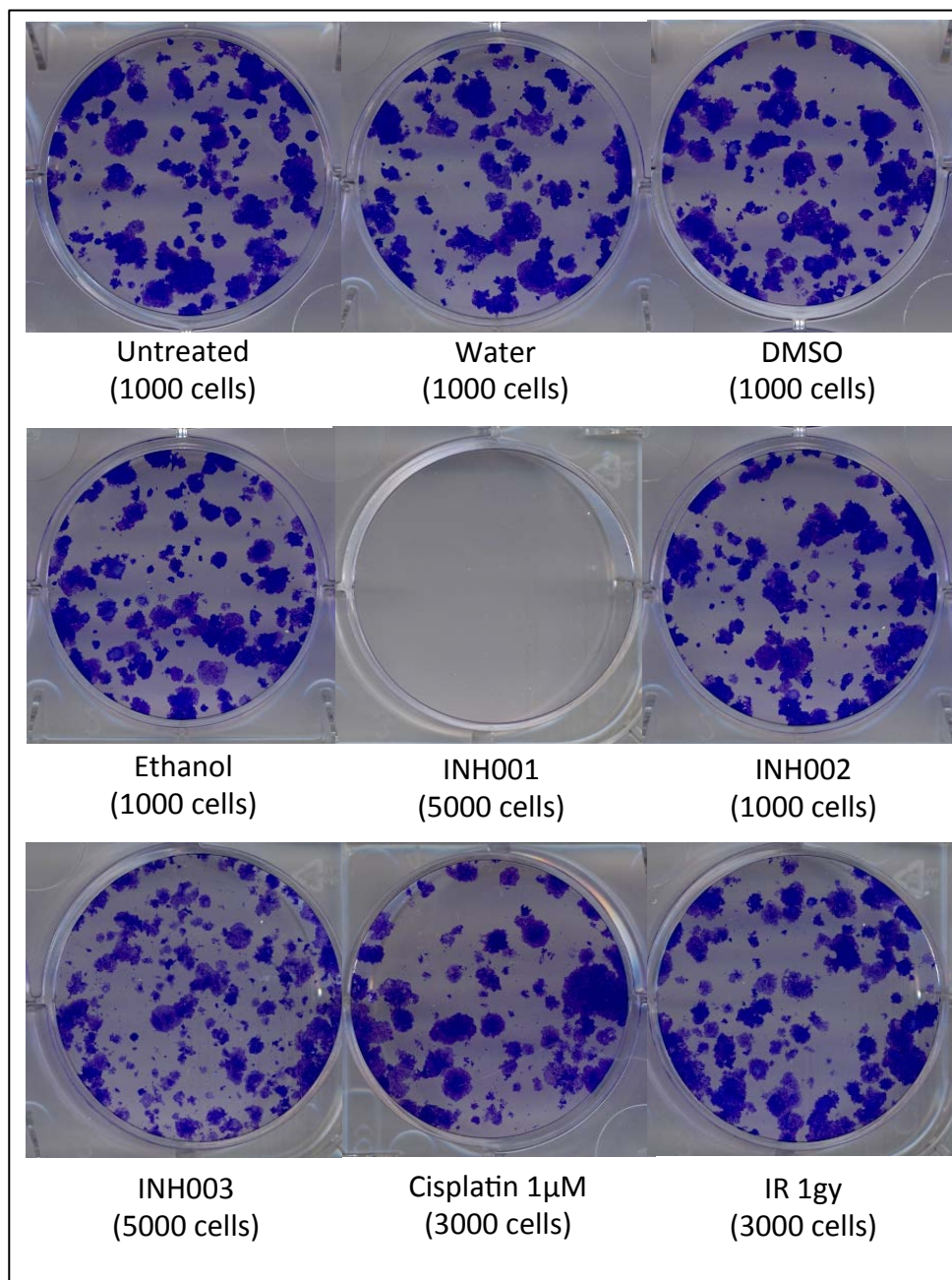
Figure 3.18: Percentage total events for each stage of the cell cycle at 72 hours treatment for VU147 cells

### 3.3.5 Clonogenic assay

#### 3.3.5.1 SCC040 cell line

The clonogenic assay was utilised to further assess the effect of drug targets on proliferation and cell survival. The required cell seeding density for each condition was predicted from previous optimisation experiments and assays. Lower seeding densities indicated no or non-significant effect on colony formation. Therefore the untreated and vehicle-only controls, which have demonstrated no effect on previous assays, were seeded at lower densities. Similarly INH002 was found to have no significant effect on the clonogenic assay, even at lower seeding densities. In contrast INH001 and INH003 showed significant effects on the assay, and required higher

seeding densities. Even at 5000 cells/well there was no visible colony formation with INH002 (Figure 3.19).



*Figure 3.19: Representative wells of clonogenic assay with SCC040 cells treated with drug targets, vehicle controls and positive controls. Individual colonies represent 50 or more cells per cluster. Cell seeding density is stated in brackets. INH001 showed no visible colony formation even at a higher cell seeding density of 5000 cell/well*

Quantitative analysis was performed to calculate the percentage survival fraction of each condition compared to the untreated control (Figure 3.20). INH002

had a non-significant reduction in survival of 21.5% (+/- 6.0, N=3). INH001 demonstrated a significant reduction of 99.5% in survival (+/- 0.5,  $p<0.0001$ , N=3), as did INH003 with 83% reduction (+/- 8.7,  $p<0.0001$ , N=3). These were similar to Cisplatin 1 $\mu$ M (82.6%, +/- 4.0,  $p<0.0001$ , N=3) and IR 1gy (75.9%, +/- 3.4,  $p<0.0001$ , N=3).

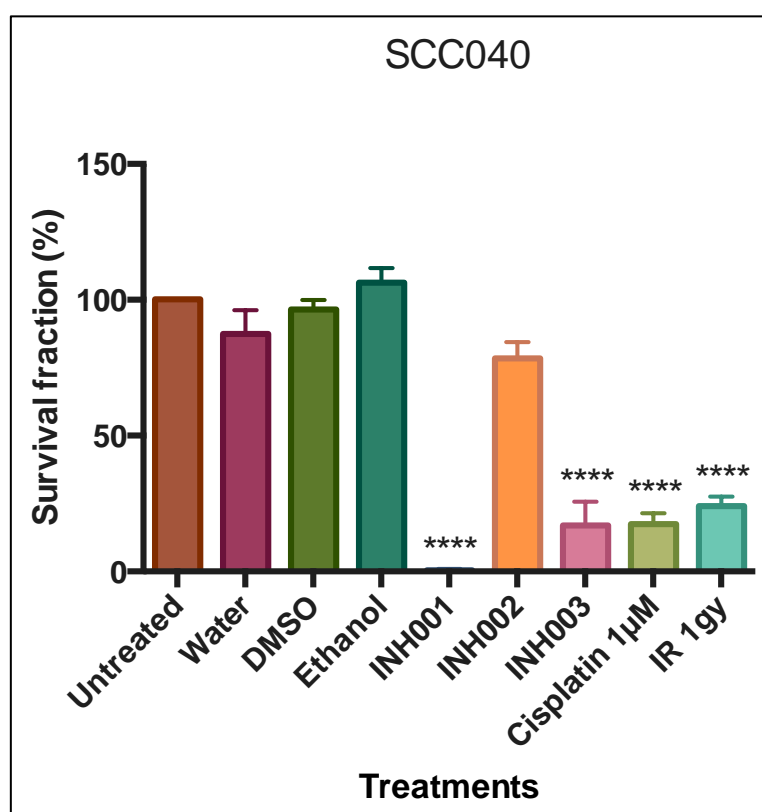
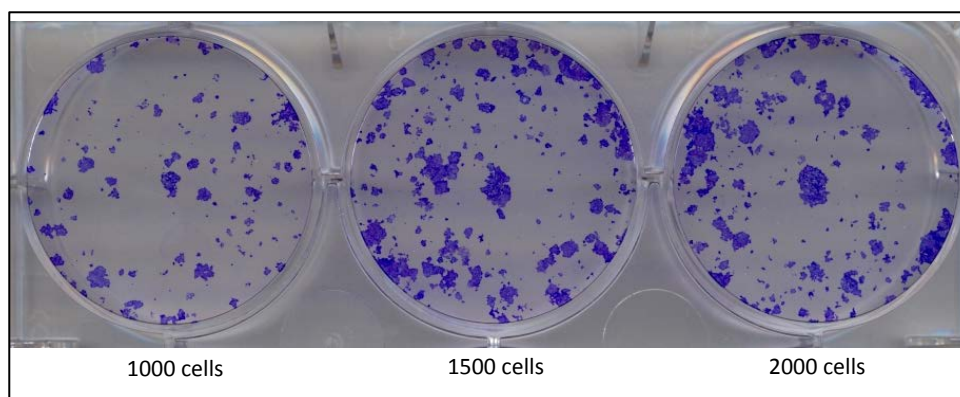


Figure 3.20: Percentage survival fraction compared to untreated control of SCC040 cells with drug targets and controls. INH001 and INH002 demonstrated significant reduction in survival fraction, comparable to Cisplatin and IR positive controls.

Statistical analysis with one-way ANOVA and post-hoc Dunnett's multiple comparisons test. (N=3; \*\*\*\* $p<0.0001$ )

### 3.3.5.2 VU147 cell line

Optimisation of the clonogenic assay with VU147 cells did not demonstrate colony formation (Figure 3.21). Therefore further testing with drug targets was not performed.



*Figure 3.21: Representative wells of untreated VU147 cells at 3 cell seeding densities. The cells did not appear to grow into colonies*

### 3.4 Discussion

The initial aim of this drug re-purposing study was to validate the results of the primary HTS of a drug library, which identified several potential drug targets including INH001, INH002 and INH003. Various assays were employed to investigate the effect of these drugs on cell proliferation, migration, cell cycle analysis and colony formation in clonogenic assays. These drug targets were tested separately on a HPV negative and a HPV positive cell line.

INH001 demonstrated a statistically significant anti-proliferative effect in SCC040 cells using the alamarBlue assay at all measured incubation time-points. This was similar to the effect of Cisplatin 5 $\mu$ M control. INH001 also significantly reduced clonogenicity and survival in the clonogenic assay with SCC040 cells, and was more effective than Cisplatin 1 $\mu$ M control. However, no significant effect was demonstrated with SCC040 cells in the migration and flow cytometry assays. Furthermore INH001 showed no effect on VU147 cells in the proliferation and flow cytometry experiments. Water was included as the vehicle-only control, and

demonstrated no effect in any of the assays. Therefore it appears that while INH001 exhibits anti-proliferative effect in both the alamarBlue and clonogenic assays, it did not demonstrate a significant effect in the flow cytometry assay compared to both INH003 and Cisplatin control. The varying seeding densities employed between these assays may partly explain this result. Both the proliferation and clonogenic experiments were seeded at lower cell densities (25,000 cells/ml and 2,500 cell/ml respectively) compared to the migration and flow cytometry assays (250,000 cells/ml and 50,000 cells/ml respectively). In fact INH001 showed the highest potency in the clonogenic assay, which was seeded at the lowest cell density of all the assays. Another aspect to consider is the effect of the drugs on the assays themselves. In the case of INH001 we experienced a change in colour of media (containing phenol red) from red to yellow on adding the drug during serial dilutions, indicating a more acidic solution. This is particularly important when utilising the alamarBlue reagent, which measures the conversion of Resazurin to Resorufin, which involves an oxidation-reduction reaction. Therefore INH001 may have interacted with alamarBlue to provide a false positive result. However, the proliferation assay included a drug-only plate for each time-point, and further analysis revealed no significant effects with INH001 on the readings. Moreover the results were standardised by subtracting the drug-only readings. The different efficacy profile of INH001 between cell lines would also suggest a true effect. The clonogenic assay provided a qualitative and quantitative method of analysing the effect of the drugs on cell survival, and confirmed the results of INH001 from the proliferation assay. The INH001 drug belongs to the class of water-soluble vitamins, which are commonly found in fruit and vegetables. Historically fruits and vegetables have been advocated by the medical profession, and are thought to be beneficial for general health and disease prevention.

An early meta-analysis of epidemiological studies investigated the relationship between fruit and vegetable intake and risk of cancer (Block et al., 1992). The study found the relative risk of laryngeal cancer was more than 2-fold with low fruit and/or vegetable consumption, and also 2-fold increase risk with oral and pharyngeal cancers. The INHANCE study (Chuang et al., 2012) found an inverse relationship between risk of HNC and consumption of fruits and vegetables (odd ratios 0.52 and 0.66 respectively). In another meta-analysis of oral cancer risk the authors found that consumption of fruit was associated with a 49% reduction in cancer risk, and 50% risk reduction with vegetable intake (Pavia et al., 2006). A recent meta-analysis found that for every increase portion of fruit and vegetables consumed per day significantly lowered all-cause mortality rate, with the maximum benefit derived from 5 portions daily (Wang et al., 2014). This supports the current 5-a-day campaign endorsed by various countries, including the United Kingdom, to encourage disease prevention.

In contrast to INH001 and INH003 the INH002 drug target appeared to have no significant effect on any of the assays, either with SCC040 or VU147 cells. Therefore it appears INH002 was a false positive result from the initial HTS. While HTS of drugs has the potential advantage of being less time consuming and resource-intensive, there will undoubtedly be several false negative and false positive results. This can be attributed to the weakness inherent in any assay utilised, as well as the arbitrary incubation period and efficacy cut-off points used to define a potential drug target. For example, the alamarBlue assay result for INH002 at 24 hours indicated a reduced proliferation of 21.9% (+/- 9.0) in SCC040 cells. Therefore INH002 can be considered a target in HTS when using an efficacy cut-off point of 26-50% reduction in proliferation. Another aspect to consider is the effect of vehicle-only control on the

assays, which was DMSO in the case of INH002. This may also cause a false positive reading during HTS. However, DMSO control did not show any significant effect in any of the assays. Although our assays show INH002 to have no true effect on both cancer cell lines, previous studies have demonstrated several anti-hypertensive drugs with re-purposing potential. An Italian study found RND-Me, an  $\alpha_1$ -adrenergic receptor ligand, demonstrated anti-proliferative properties against a panel of cancer cell lines including HNC cells (Zagni et al., 2015). In another study 3 commercially available anti-hypertensive drugs (Lisinopril, Propranolol, Nifedipine) were tested in-vitro against various cancer cell lines, and also tested on EACC (Ehrlich Ascites Carcinoma Cell) in an animal model (El Sharkawi et al., 2013). At 3000 $\mu$ g/ml concentration Propranolol reduced EACC growth by 97.7%, Lisinopril by 18.28%, and Nifedipine by 11.40%.

Among our 3 drug targets INH003 demonstrated the greatest effect across the assays and in both cell lines. INH003 showed a statistically significant anti-proliferative effect on SCC040 cells at all measured time-points. In the VU147 cells the anti-proliferative effect was significant at 48 and 72 hours only. This result was similar to the Cisplatin 15 $\mu$ M control in both cell lines. The flow cytometry experiment in SCC040 cells at 72 hours found INH003 to exert a significant effect on cell cycle with a peak in Sub G1, which was similar to the Cisplatin control. However, this effect was not demonstrated in the flow cytometry assay of VU147 cells at 16 and 72 hours. The clonogenic assay with SCC040 cells provided further evidence of INH003 potency, with a statistically significant reduction in colony formation similar to Cisplatin and IR controls. The vehicle-only control, Ethanol, showed no significant effect on any assay. These results validate the initial HTS, and



were more consistent across the assays compared to INH001. The effect of INH003 appears more pronounced in the SCC040 cells compared to VU147 cells, and this will be discussed later. Previous studies have discussed the potential use of anti-helminthic drugs in cancer treatment. An interesting case report from Sweden described the treatment of a 74-year old male patient with refractory metastatic colon cancer (Nygren et al., 2014). The patient was initially treated with radical surgical resection and post-operative chemotherapy, but metastatic disease progression was observed. The authors had previously identified Mebendazole during in vitro drug re-purposing experiments using colon cancer cells. Thus the patient was consented for Mebendazole treatment, and after six weeks a repeat CT scan revealed almost complete disease remission. This case highlights the potential of drug re-purposing in a real clinical setting. Ideally cancer therapies should display high efficacy and selectivity, as do the anti-helminthic drugs. Elucidating a common pathway between cancers and parasitic infections may identify potential drug targets, and provide an insight into their mechanism of action.

Cisplatin was included as a positive control, which is the current standard chemotherapy treatment in HNC. We found a significant effect of Cisplatin on both cell lines, which was reproducible in all the assays. However, Cisplatin controls only demonstrated a significant effect on proliferation at 48 and 72 hours in both cell lines. In the migration assay the Cisplatin controls all showed an effect on wound closure, with Cisplatin 10 $\mu$ M and 15 $\mu$ M being statistically significant. There was no significant effect on the flow cytometry cell cycle analysis at 16 hours, but at 72 hours an abnormal cell cycle pattern was demonstrated in SCC040 cells and G2/M cell

cycle arrest in VU147 cells. The clonogenic assay also showed a statistically significant reduction in SCC040 cell colony formation with Cisplatin control.

Although only 2 assays (alamarBlue and flow cytometry) contained both cell lines for comparison, we found a difference in drug effect profiles between SCC040 and VU147 cells. The SCC040 cells appeared to demonstrate more susceptibility to INH001 and INH003, as well as Cisplatin control. While INH001 demonstrated a significant effect on proliferation of SCC040 cells, no effect was seen in VU147 cells. INH003 also demonstrated significant effect on SCC040 cell proliferation at all incubation time-points, but only at 48 and 72 hours in VU147 cells, albeit less pronounced. For example, at 72 hours with INH003 there was a 93.2% ( $p < 0.0001$ ) reduction in proliferation with SCC040 cells compared to 85.9% ( $p < 0.001$ ) with VU147 cells. INH003 exhibited a significant effect on SCC040 cells in the flow cytometry assay, but no measurable effect on VU147 cells. The Cisplatin control also demonstrated a variation between cell lines. For example, Cisplatin 5 $\mu$ M reduced SCC040 cell proliferation by 82.7% ( $p < 0.0001$ ) at 72 hours, but had a non-significant reduction of 61.2% in VU147 cells. Flow cytometry analysis at 72 hours showed a significant effect of Cisplatin in SCC040 cells, while VU147 cells demonstrated a less significant increase in Sub G1 area and G2/M peak. The varying results could be attributed to the HPV status of the chosen cell lines; SCC040 cells are HPV negative and VU147 cells HPV positive. The SCC040 cells also contain wild-type p53, whereas the HPV positivity of VU147 cells is likely to disrupt the p53 pathway (Figure 1.7). As previously discussed, p53 is known as a tumour suppressor gene product and has numerous cellular roles, including cell cycle arrest and senescence (Figure 3.22). Therefore the SCC040 cells containing functional p53 are likely to

respond to potential drug targets by inducing cell cycle arrest and/or apoptosis. However, the VU147 cells containing HPV oncoproteins would disrupt these pathways and encourage cellular immortalisation.

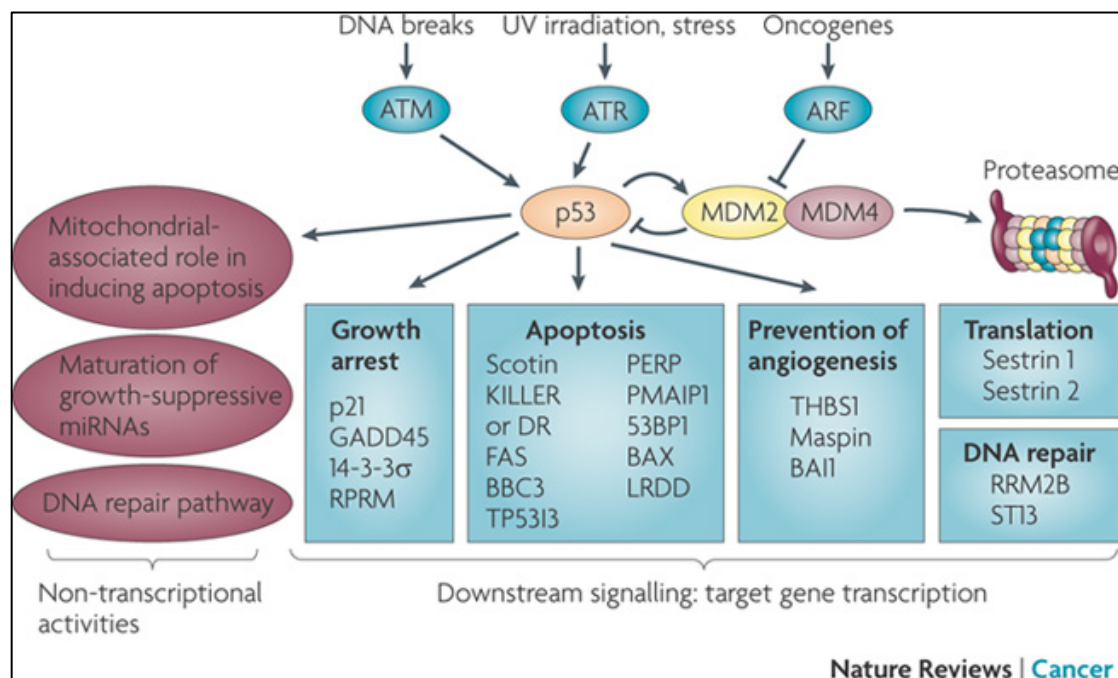


Figure 3.22: p53 cellular pathways and functions (Brown et al., 2009)

There are several inherent limitations to our study that we have considered when designing the study and when interpreting the results of the current study. Firstly, initial identification of target drugs with HTS was defined by the effect of proliferation compared to untreated control. However, while proliferation is a key characteristic of cancer cells there are other pathological traits, such as invasion, metastasis and angiogenesis. These are known as the hallmarks of cancer (Hanahan et al., 2000). Therefore initial HTS defined by anti-proliferation effects is likely to exclude other potential targets with anti-cancer properties. However, without an automated and cost-effective alternative the cell viability assays are ideal for HTS. Furthermore any HTS tool will return a number of false positive and false negative results. In the case of false positive targets this can lead to ‘dead-ends’ during validation, which is particularly important with time and resource constraints. The

assay results of true positive targets can help refine the HTS and better predict which potential drug target will be successful. Another limitation was the selection of cell lines. The SCC040 cell line was ideal due to its' commercial availability, HPV negative status and ability to grow successfully in vitro. In contrast the VU147 cell line was non-commercial and could not be optimised in the migration and clonogenic assays. Initially another HPV positive cell line (SCC154) was purchased but did not provide satisfactory growth results in cell culture. Optimisation of SCC154 cells or sourcing an alternative cell line would provide an ideal candidate to compare against the SCC040 cells. The various assays employed during this project will obviously have their own limitations. For example, the limits to the alamarBlue assay were discussed earlier. However, the optimisation of each assay prior to application of drug targets helped to ensure improved accuracy and reproducibility.

The results of our study have identified both INH001 and INH003 as potential drug targets and warrant further investigations. Additional assays could be utilised to elucidate the mechanism of action of these targets. For example, apoptosis can be measured with Caspase 3/7, Annexin V/ PI or TUNEL assays. An invasion assay may also help to elucidate the inhibitory effects of target compounds on cancer cell metastatic properties. Identifying more drug targets can be achieved by screening other drug libraries and on-going literature searches can be used to put together unique collections of drugs that exhibit promising activity in other types of cancers. The INHANSE group has already begun investigations into combination of drug compounds with standard treatments, such as Cisplatin and IR. These may yield even more potent treatment results in a potentially synergistic manner. Animal models and

experiments on primary cell culture are also underway, and the outcomes will enable progression into Phase 3 of the Accelerated project.

## **Chapter 4: Conclusions and Future Studies**

Despite great advances in our understanding of disease pathogenesis and treatments cancer remains a significant burden, with profound effects on patient morbidity and mortality. An evolving principle is the concept of personalised and tailored management of cancer patients, in order to improve outcomes and prognostic accuracy. However, this requires a greater understanding of the relationship between tumour and patient characteristics, as well as improved treatment options. The main objectives of this thesis were to investigate the survival outcomes of oropharyngeal cancer (OPC) according to a combination of HPV and p16 status, and to evaluate the drug re-purposing potential of three drug targets (INH001, INH002, INH003) using HNC cells lines. Firstly, a systematic review and descriptive analysis was conducted to assess the prognostic significance of HPV/p16 in OPC, with particular interest in the importance of the discordant groups. Secondly, various laboratory assays were utilised to assess the effect of the three drug targets on HPV negative (SCC040) and HPV positive (VU147) cell lines.

### **4.1 HPV+/p16+ has the best prognostic outcome and HPV-/p16- has the worst**

Previous studies and meta-analysis have confirmed that HPV positive OPC show significantly better prognostic outcomes compared to HPV negative tumours (Ragin et al., 2007b, Dayyani et al., 2010, O'Rorke et al., 2012). The results of this systematic review corroborate these findings. Combining HPV and p16 status, the best prognostic group was HPV+/p16+ (true positive). Both at 3 and 5-year time points the HPV+/p16+ cohort had the most favourable DFS, DSS and OS rates compared to the other HPV/p16 groups. Furthermore HPV+/p16+ conferred the

lowest HR values of all the groups. In contrast the HPV-/p16- cohort had the lowest 3 and 5-year DFS, DSS and OS rates, as well as the least favourable HR values.

There were differences in demographics and characteristics between these two groups. Although both cohorts had a greater proportion of male subjects, the HPV+/p16+ displayed even more male preponderance. The HPV+/p16+ also showed a lower average mean age compared to HPV-/p16-. The HPV-/p16- cohort contained a greater proportion of smokers and excessive drinkers. However, the HPV+/p16+ group comprised a larger percentage of grade IV tumours, and was more likely to be poorly differentiated cancers. The HPV+/p16+ group was also more associated with tonsil or base of tongue tumours.

The findings of this review support the notion that HPV-driven cancers are likely to represent a different disease entity, with unique pathophysiology and tumour characteristics (Pyrri et al., 2011). Previous authors have utilised HPV status for prognostic modelling (Ang et al., 2010). While true positive and true negative tumours represent the vast majority of cases, the discordant groups were the main focus of this review.

## **4.2 HPV-/p16+ has a better prognostic outcome compared to HPV+/p16-**

Previous studies have shown that the discordant groups, HPV+/p16- and HPV-/p16+, represent a minority of cases (see Table 1.1). This review confirms these findings, with both discordant groups representing 20% of the total cases. This



proportion was more than the example studies represented in Table 1.1. Comparing between the discordant groups the HPV-/p16+ showed more favourable 3 and 5-year DFS and OS rates. Furthermore the HPV-/p16+ demonstrated lower HR values compared to the HPV+/p16- group, albeit with limited data availability.

There were also differences between group characteristics. The HPV-/p16+ cohort demonstrated a lower average mean age and a greater male percentage compared to the HPV+/p16- group. Both groups demonstrated similar proportion of smokers, but the data for alcohol consumption was unavailable for HPV-/p16+. All the HPV-/p16+ cases were either tonsil or base of tongue, whereas the HPV+/p16- contained a higher proportion of other oropharyngeal sites. Finally, the HPV-/p16+ cohort showed a higher percentage of stage IV tumours compared to HPV+/p16-.

It appeared that the HPV-/p16+ cohort had similar group characteristics compared to the true positive cohort, whereas the HPV+/p16- group correlated with the true negative cohort. Prognostically, while the true positive group demonstrated the best survival outcomes the next best survival rates were seen in the HPV-/p16+ cohort. Therefore it would appear from this systematic review that HPV-/p16+ status confers a better prognostic outcome compared to the HPV+/p16- discordant group. However, statistical significance could not be determined due to the limited data available.

The significant bias in the review results relates to the differences between HPV and p16 testing among the included studies. Ideally this would have been explored further if a meta-analysis were possible, by use of sensitivity analysis.

Therefore while the descriptive analysis has provided an insight into the differences between the HPV/p16 subgroups the results remain inconclusive.

### **4.3 INH001 and INH003 show promising drug re-purposing potential**

Three drug targets were chosen for further analysis in this study following the initial high-throughput screening. The results confirmed the potential of INH001 and INH003 as drug re-purposing agents. INH001 showed a statistically significant effect on both the proliferation and clonogenic assays using the SCC040 (HPV negative) cell line. In comparison there was no significant effect with INH001 using the VU147 (HPV positive) cell line. However, INH003 demonstrated a statistically significant effect on the proliferation assay using both these cell lines. Furthermore INH003 showed a statistically significant effect on both the flow cytometry and clonogenic assays using the SCC040 cell line. In contrast INH002 was found to have no significant effect across all the assays and cell lines.

Based on the data of this study both INH001 and INH003 would be ideal targets to continue further testing as part of the Accelerated project (Figure 3.1). In fact, the results of the assays have shown INH003 to be at least as effective on the two cell lines compared to Cisplatin, which is the current standard chemotherapy agent. These results are both exciting and promising for future cancer therapy, and has demonstrated the potential of the Accelerated project to deliver novel cancer treatments. There are many other drug targets to be tested, and numerous HNC cell lines to be utilised. Furthermore this system, if successful, can be easily adapted to discover therapies in other cancers. Ideally these would deliver new and effective

treatments, which could be both beneficial for patient outcomes and financially attractive.

#### **4.4 Target drugs more effective in the HPV negative (SCC040) cell line**

The results of the assays demonstrated a difference in drug target effect between the cell lines. While INH001 and INH003 significantly effected proliferation in the SCC040 cell line, only INH003 had a significant effect on the VU147 cell line. However, the effect of INH003 was not as pronounced using the VU147 cell line. Furthermore the Cisplatin control also showed less effect on VU147 cells, albeit still statistically significant. This would suggest the VU147 cells were more resistant to these agents. The role of HPV in disease pathogenesis and cellular mechanisms with regards to the results is unclear. Further testing with other HPV positive and negative cell lines would be required to investigate this. However, one major difficulty encountered with the VU147 cells was in optimisation for the assays. The migration and clonogenic assays could not be applied to the VU147 cells due to the results of the optimisation experiments. Understandably some cells lines do not exhibit the characteristics required for in-vitro testing. Again, testing with other HPV positive cell lines may provide further results.

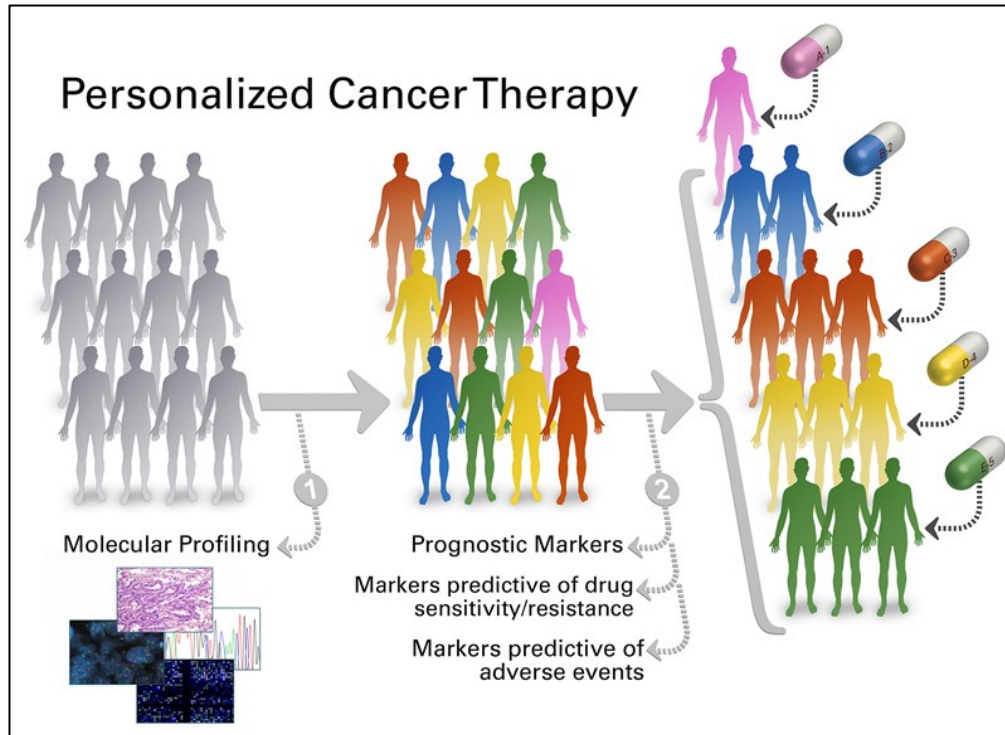
#### **4.5 Implications for personalised treatment and prognosis**

As research into cancer mechanisms and therapies continues to expand the key component of success is in translational research. The vast majority of basic science

discoveries rarely impact on real life patients. For example, only 1 in 5000 new drug discoveries are eventually marketed (MedicineNet, 1999). In order to improve the success and effectiveness of research greater emphasis on personalised therapy is required. Just as we are individuals, the course of any disease is unique between people, with patient-related factors and disease-related factors interacting. Treatments based on 'one-size fits all' are likely to be less effective.

The results of systematic review and drug re-purposing study demonstrate the potential to focus on personalised management. Biomarkers, genetic studies and risk factors of disease can help predict the risk of specific cancers and prognostic outcomes. Furthermore both patient and disease-related characteristics can be combined to predict the most effective treatment strategy. The future model of cancer management would involve an individual and tailored approach. For example, patients would initially attend a specialist clinic where numerous blood tests, imaging and tissue sample extractions are performed. Tissue samples would undergo laboratory testing, including molecular and genetic profiling. A panel of biomarkers would also be tested. The results of all these tests can then be integrated into a prognostic model, which can predict survival and potential therapeutic response (Figure 4.1). Furthermore, patients will have tailored therapy based on the AccelerATED model of testing, involving primary cell cultures and mouse models. Target drugs and combination therapies will be selected for the patient, and response to treatment monitored by the multidisciplinary team (i.e. clinician, radiologist, pathologist, scientist etc.). Combining effective drug targets, radiotherapy and surgery would provide the best prognostic outcomes, with improved patient quality of life.

Depending on the prognostic models patients would either undergo aggressive treatment, or de-escalation therapy in order to ensure the best outcomes.



*Figure 4.1: Example of the stages for personalised cancer therapy (MD-Anderson, 2015)*

## 4.6 Future studies

The systematic review of OPC has provided an interesting insight into the prognostic potential of combining HPV and p16. The results could be improved by the addition of high quality RCTs, if possible, in future reviews. Additionally a consensus on HPV and p16 testing is required to improve the accuracy of the data and minimise bias. Given the current data available an IPA (individual patient analysis) can be performed with the studies included in this review, and also include studies excluded from the systematic review due to lack of data. This is considered the ‘gold-standard’ for systematic reviews, and should also enable a meta-analysis.

Further studies on the impact of HPV in OPC are required. In particular the exact pathogenesis of HPV-driven cancer in head and neck sites needs to be elucidated. Epidemiological studies are on going, and the future trend of OPC incidence would be particularly interesting given the changing demographics and disease characteristics. The impact on HPV vaccination employed in several countries also merits further studies.

The work on the Accelerated project is on going with the InHANSE group. The results of the drug screening and validation assays will be further tested in mouse models and primary cell cultures. The imminent opening of ITM (Institute of Translational Medicine) in Birmingham will help to propel this and many projects, and ultimately improve patient outcomes.

The future prospects for cancer patients certainly appear to be positive, with many exciting new discoveries and innovations. While curing cancer may never be possible, each breakthrough brings us closer to that reality.

## Appendix

### (Quality Assessment Scores)

#### Reviewer 1:

Study	Study Participation	Study Attrition	Prognostic Measure	Outcome Measure	Confounding Measure	Analysis	Total score
Evans et al	2	1	2	2	1	2	<b>10</b>
Hong et al	2	1	2	1	1	2	<b>9</b>
Junor et al	2	1	1	1	1	2	<b>8</b>
Lewis et al	1	1	2	1	1	1	<b>7</b>
Song et al	0	1	1	1	0	1	<b>4</b>
Stephen et al	0	1	1	0	1	1	<b>4</b>
Weinberger et al	1	1	2	1	1	2	<b>8</b>

#### Reviewer 2:

Study	Study Participation	Study Attrition	Prognostic Measure	Outcome Measure	Confounding Measure	Analysis	Total score
Evans et al	2	2	2	2	2	2	<b>12</b>
Hong et al	2	1	1	1	1	2	<b>8</b>
Junor et al	2	1	1	2	1	2	<b>9</b>
Lewis et al	1	1	1	1	1	2	<b>7</b>
Song et al	1	1	1	1	0	1	<b>5</b>
Stephen et al	1	1	1	0	1	1	<b>5</b>
Weinberger et al	1	0	2	1	1	2	<b>7</b>

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